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Identification of Bromodomain-Containing Protein 4 (BRD4) as a Novel Marker and Epigenetic Target in Systemic Mastocytosis and Mast Cell Leukemia

Ghaith Wedeh

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THESE DE DOCTORAT
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préparée à l'**Ecole Normale Supérieure de Cachan**

ÉCOLE DOCTORALE N° 582
Cancérologie: Biologie, Médecine, Santé

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Par

Mme Ghaith Wedeh

**Identification de BRD4 comme nouvelle cible thérapeutique dans le traitement
des mastocytoses systémiques agressives et des leucémies à mastocytes**

Thèse présentée et soutenue à Cachan, le 29 Janvier 2016:

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*“What we learn with pleasure we never forget”
Alfred Mercier*

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LIST OF ABBREVIATIONS

A	adenine
17-AAG	17-demethoxygeldanamycin
ABL	Abelson murine leukemia viral oncogene homolog
AEC	sodium acetate
Ag	antigen
ALL	acute lymphoblastic leukemia
Allergin1	allergy inhibitor receptor 1
allo-HSCT	allogeneic hematopoietic stem cell transplantation
AML	acute myeloid leukemia
APC	antigen-presenting cell
APC	allophycocyanin
APRIL	a proliferation-inducing ligand
ATRA	all-trans-retinoic acid
ASDs	autism spectrum disorders
ASM	aggressive SM
ASXL1	additional sex combs-like 1
ATP	adenosine triphosphate
Bas	basophils
BAZ1B	bromodomain adjacent to zinc finger domain protein 1B
BCL-2	B-cell lymphoma-2
BCL10	B cell lymphoma 10
BD	Becton Dickinson
BD1/BD2	bromodomain tandems
BET	bromodomain and extra C-terminal domain
BIM	Bcl-2 (B-cell lymphoma-2) interacting modulator of cell death
BLYS	B-lymphocyte stimulator factor
BM	bone marrow
BMMCs	bone marrow derived mast cells
BP	blocking peptide
BRD	bromodomain
BRD4	bromodomain containing protein 4
BRDT	BRD testis-specific form
BRD4S	bromodomain containing protein 4 short form
BRD4L	bromodomain containing protein 4 long form
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
C	cytosine
CAD	coronary artery disease
C3aR	receptor for complement component 3a
C5aR	receptor for complement component 5a
C3b	complement component 3b
C3biR	receptor for complement component 3bi
CB	cord blood
CCR	CC-chemokine receptor
CD	cluster of differentiation
2-CdA	cladribine (2-chlorodeoxyadenosine)
CEREMAST	French Reference Center for Mastocytosis

CHD4	chromodomain helicase DNA-binding protein 4
CHO	chinese hamster ovary
CI	combination index
CMML-eo	CMML with eosinophilia
CNS	central nervous system
CPA	carboxypeptidase A
CTMC	connective tissue mast cell (in mice)
CtR	complement receptor
CR	complete remission
CRAC	calcium release-activated calcium
CRH	corticotropin-releasing hormone
CrkL	Crk (CT10 regulation of kinase) -like protein
CSF-1	colony-stimulating factor-1
CTM	c-terminal motif
CXCR	CXC-chemokine receptor
CXCL8	CXC-chemokine ligand 8
cys-LT	cysteinyl (cys) leukotrienes (LTs)
D	domain
DAG	diacylglycerol
DC	dendritic cells
Del	deletion
DENV	dengue virus
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dok-1	downstream of tyrosine kinase 1
DTT	dithiothreitol
EAE	experimental allergic encephalomyelitis
ECD	extracellular domain
ECM	extracellular matrix
ECM	extracutaneous mastocytoma
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-to-mesenchymal transition
E-NPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3
ER	endoplasmic reticulum
ER α	estrogen receptor α
EREs	estrogen response elements
ERK	extracellular signal regulated kinase
ERr	estrogen receptor
ET	extra-terminal domain
FAD	Food and Drug Administration
FAS	Apoptosis stimulating fragment
Fc ϵ RI	the highly affinity receptor for IgE
FCS	fetal calf serum
Fc γ RI	high affinity receptor for IgG
Fc γ RII	low affinity receptor for IgG
Fc γ RIII	low affinity receptor for IgG
FES	feline sarcoma oncogene
FGF2	fibroblast growth factor 2

FITC	fluorescein isothiocyanate
FLT3	fms-like Tyrosine Kinase-3
FOXO3a	forkhead transcription factor (forkhead box, class O3A)
F protein	Fusion protein
Fsh	female-sterile homeotic
Fshrg1	female sterile homeotic related gene 1
G	guanine
Gab2	Grb2 associated binding protein 2
Gads	Grb2 related adaptor downstream of Shc
GAGs	glycosaminoglycans
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN5L2	general control of amino acid synthesis protein 5-like 2
GFP	Green Fluorescent Protein
GI	gastrointestinal
GIST	gastrointestinal stromal tumors
GCs	glucocorticosteroids
GLTSCR1	glioma tumor suppressor candidate region gene 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP49B1	glycoprotein 49B1
Grb2	growth factor receptor bound protein 2
GRK2	G protein-coupled receptors GPCR kinase 2
GRK2-RH	GRK2-RH (regulator of G protein signaling homology domain
GVHD	graft-versus-host disease
HATs	histone acetyltransferases
HBSS	Hank's balanced salt solution
HDACs	histone deacetylases
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
HMC-1	human mast cell line
HPV	human papillomavirus
H1R/H2R	histamine receptor
HRB	histamine release buffer
HRP	horseradish peroxidase
HRPT	hypoxanthine guanine phosphoribosyltransferase
hsp90	heat shock protein 90
HU	hydroxyurea
HZ4	Hardy-Zuckerman 4
ICAM	intercellular adhesion molecule receptor
ICC	immunocytochemistry
Ig	immunoglobulin
IgE	immunoglobulin E
ICH	immunohistochemistry
IL-4	interleukin-4
IL-17	interleukin-17
IFN- α	interferon alpha
IFN- γ	interferon gamma
IFN- γ R	interferon gamma receptor
IP3	inositol 1,4,5-triphosphate

IPMN	intraductal papillary mucinous neoplasia
ISM	indolent SM
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IV	intravenously
JAK	Janus kinase
JMD	juxtamembrane domain
JNK	c-Jun N-terminal kinase
JMML	juvenile myelomonocytic leukemia
K _{Ac}	acetylated lysine
KD	kinase domain
kDa	kilo dalton
KID	kinase insertion domain
KL	KIT ligand
LAB	linker for activated B cell
LAMP-3	lysosome-associated membrane glycoprotein 3
LAT	linker for activation of T cells
LIRs	leukocyte immunoglobulin-like receptors
LFA-2	leukocyte function antigen-2
LPA	lysophosphatidic acid
LPCs	lymphoplasmacytic cells
LPS	lipopolysaccharide
LT	large T antigen
LTC4	leukotriene C4
LTD4	leukotriene D4
MAb	monoclonal antibodies
MAC	myeloablative conditioning
MAFA	mast-cell-function-associated antigen
MAIR-1	myeloid-associated immunoglobulin-like receptor
MALT10	mucosa-associated-lymphoid-tissue lymphoma-translocation protein 10
MAPKs	Ras/Raf/mitogen-activated protein kinases
MC	mast cell
MCAS	MC activation syndrome
MCC	MCs containing chymase and cathepsin G
MCL	mast cell leukemia
MCMRS	MC mediator-release symptoms
MCPV-1	mast cell progenitor Vienna (1-4) cell lines
MCS	mast cell sarcoma
M-CSF	macrophage colony-stimulating factor
MCT	MC containing only tryptase
MCTC	MC containing tryptase, chymase, carboxypeptidase and cathepsin G,
MCV	merkel cell polyomavirus
MDS	myelodysplastic syndrome
MDSCs	myeloid-derived suppressor cells
MEK	mitogen-activated protein kinase
MGF	MC growth factor
MGG	May-Grünwald Giemsa

MIRR	multichain immune recognition receptor
MLL	mixed lineage leukemia
MM	multiple myeloma
MMC	mucosal mast cell (in mice)
mMCP	mouse mast cell protease
MNCs	mononuclear cells
MPD	myeloproliferative disorder
MR	major response
MS-5	murine stromal cell line
NaCl	sodium chloride
NPC	nasopharyngeal carcinoma
NCT	National Clinical Trial
n.d.	not determined
Nd:YAG	neodymium-doped yttrium aluminium garnet
NFAT	nuclear factor for T cell activation
NGF	nerve growth factor
NMC	NUT midline carcinoma
NMR	nuclear magnetic resonance
NSAIDs	nonsteroidal anti-inflammatory drugs
NSCLC	non-small cell lung cancer
NSG mice	NOD/SCID IL-2R γ ^{-/-} mice
NT	neurotensin
NTAL	non- T cell activation linker
NuRD	nucleosome remodeling complex
ORAI1	store-operated Ca ²⁺ channel
ORR	overall response rate
OS	overall survival
Q-PCR	quantitative polymerase chain reaction
PAF	platelet activating factor
PAR2	protease-activated receptor2
PB	peripheral blood
PBS	phosphate-buffered saline
PDA	pancreatic ductal adenocarcinoma
PDGF	platelet derived growth factor
PDK1	phosphoinositide-dependent kinase
PE	phycoerythrin
PEL	primary effusion lymphoma
PFS	progression-free survival
PGD2	prostaglandin D2
PGN	peptidoglycan
Pgp1	phagocyte glycoprotein 1
PHD	plant homeodomain
PI3K	phosphatidylinositol 3-kinase
PID	P-TEFb-interacting domain
PI	propidium-iodide
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5,-triphosphate (PI(3,4,5)P3)

PIR-B	paired Ig-like receptor B
PKB	protein kinase B
PKC	protein kinase C
PLA2	phospholipase A2
PLC- γ	phospholipase C gamma
PLD	phospholipase D
PMA	phorbol myristate acetate
PTD	phosphotransferase domain
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTEFb	positive transcriptional elongation factor complex
PTK	protein-tyrosine kinase
PTMs	post-translational modifications
PTP	PTK-protein tyrosine phosphatase
PUVA	psoralen plus ultraviolet (UV) A
PWWP	pro-trp-trp-pro motif
RBL-2H3	rat basophilic leukemia cell line
Rh	recombinant human
RIA	radioimmunoassay
RING3	really interesting new gene 3
RNA	ribonucleic acid
RNAi	RNA interference
RGS13	regulator of G protein signaling (RGS) 13
RRP1B	ribosomal RNA processing 1 homolog B
RSV	respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SEED	serine–glutamate–aspartate-rich region
SF	steel factor
SFM	serum free media
SH2	Scr-homology 2
Shc	Src homology 2 (SH2)-domain-containing transforming protein C
SHIP	Src homology 2 (SH2) domain-containing inositol 5'-phosphatase
SHP-1	Src homology region 2 domain-containing phosphatase-1
ShRNA	small hairpin RNA
SIPA1	signal-induced proliferation associated protein 1
SiRNA	small interfering RNA
SIRP α	signal regulator protein α
SIRT6	sirtuins
SK	sphingosine kinase
SLP76	SH2-containing leukocyte-specific protein of 76 kDa
SM	systemic mastocytosis
SM-AHNMD	systemic mastocytosis associated with another hematological non MC disease
SNAREs	soluble N-ethyl-maleimide-sensitive factor (NSF) attachment protein receptors
SMARCA5	switch mating type/sucrose non-fermenting-related matrix-associated actin-dependent regulators of chromatin subfamily A
SOCS-1	suppressor of cytokine signaling
S1P	sphingosine 1-phosphate

SPA-1	signal-induced proliferation-associated protein 1
SSc	systemic sclerosis
SSM	smoldering SM
STAT	signal transducer and activator of transcription
STIM1	stromal interaction molecule 1
SU2	Sad1 and UNC84 containing 2
Syk	spleen tyrosine kinase
T	thymine
TAFs	TBP-associated factors
TEC	tyrosine kinase expressed in hepatocellular carcinoma
TET2	ten-eleven translocation
Tf-R	transferrin receptor
TGF- β	transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TKR	tyrosine kinase receptor
TMD	transmembrane domain
TPO	thrombopoietin
TMEP	telangiectasia macularis eruptiva perstans
TKR	tyrosine kinase receptor
TRAIL	tumor necrosis factor related apoptosis-inducing ligand receptor
Treg	regulatory T
TRIMs	tripartite motif-containing proteins
TRM	treatment-related mortality
TRPC1	transient potential Ca ²⁺ channel 1
TSPAN-29/p24	tetraspanin-29/platelet antigen p24
Ucn	urocortin
URD	unrelated donors
VCAM1	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
v-myc	myelocytomatosis viral oncogene
WBCs	white blood cells
WDSM	well-differentiated systemic mastocytosis
WHO	World Health Organization
WM	Waldenström macroglobulinemia
WT	wild type

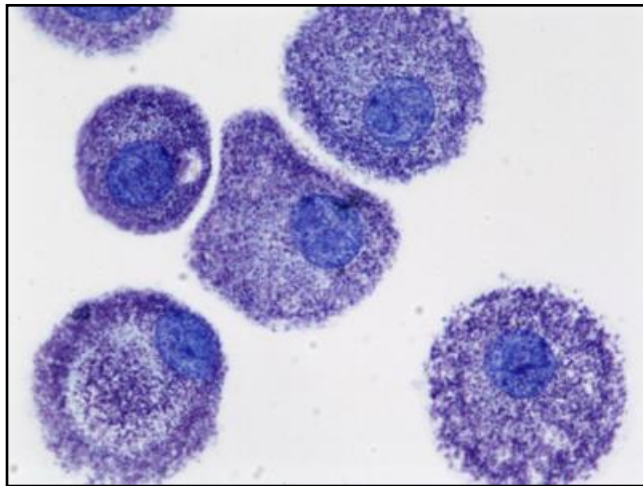


Figure 1: Metachromatic granules of peripheral blood (PB)-derived human MCs visualized after toluidine blue staining (Figure from Metcalfe *et al.* 2008).

INTRODUCTION

1. Mast cells

1.1. Historic overview

Mast cells (MCs) are long-lived, multifunctional, tissue dwelling granulated immune cells that had been first described by Paul Ehrlich (1854-1915) in 1878. Ehrlich reported MCs that he found in connective tissues as cells containing basophilic granules that can be metachromatically stained in red with aniline dye. He coined for these aniline-reactive granular cells the term “mastzellen” (in German), that means well-fed cells, in the belief that the large amount of granules they contain might develop as a result of hypernutrition (Ehrlich 1878). The word “mast” may be derived from German “mästen” (from masticate), or from Greek, which means breast. Later, Ehrlich with Westphal (one of his pupils) reported the solubility the MC granulations in water (Westphal 1891). Ehrlich also got Nobel Prize in 1908 for his observations about MCs and their localization in contact with nerve fibers, blood vessels and gland canals.

Paul Ehrlich’s observations are considered as a milestone in the study of MC biology and MCs have later become the subject of numerous investigations and receive today increasing attention. At Ehrlich’s time, the origin of MCs was obscure as well as their heterogeneity, their biological roles or the structure and the composition of their metachromatic granulations. MCs are identified by the presence of metachromatic granules in their cytoplasm, when stained with toluidine blue (Figure 1). The prominent cytoplasmic granules of MCs are rich in sulfated glycosaminoglycans (GAGs) that are responsible for their metachromatic staining by basic dyes such as toluidine blue and aniline dyes. These acidic amino sugars consist predominantly of chondroitin sulfates.

MCs are found at the interface between the host and the external environment such as skin, gastrointestinal (GI) tract and respiratory mucosa (Ehrlich 1878; Metcalfe *et al.* 1997; Jamur 2005; Galli *et al.* 2005; Metcalfe & Boyce 2006). In 1937, almost 60 years after Ehrlich's description of MCs, Holmgren and Wilander reported that tissues containing large number of MCs are particularly rich in heparin (Holmgren & Wilander 1937). Ten years later, it was shown that both histamine and

heparin are released from dog liver during anaphylactic shock (Rocha *et al.* 1947). Jorpes reported for the first time that heparin is produced in all species by tissue MCs and that for certain tissues there is a good correlation between the histamine content and the heparin content (Jorpes 1946). A few years later, Riley and West reported that MCs have an important content of histamine, and play a possible role in histamine release in tissues (Riley & West 1953).

The heterogeneity of MCs was first reported by Enerbäck in the 60s (Enerbäck 1966). He showed that rat MCs in intestinal mucosa could not be identified on histological sections because of their sensitivity to routine formalin fixation in contrast to skin MCs. These mucosal MCs stained blue after suitable fixation and staining with Alcian blue and safranin, whereas the connective tissue MCs stained red with safranin (Enerbäck 1966; Enerbäck 1986). William Bate Hardy also contributed to the shaping of the concept of “heterogeneity of MCs”, since he focused on the ‘wandering cells’ in different mammalian species and distinguished two types of granular cells, corresponding today to the connective tissue-type MCs and mucosal-type MCs. He also described the degranulation of rat MCs (Kanthack & Hardy 1894; Hardy & Wesbrook 1895). In humans, MCs are regionally heterogeneous in terms of content in proteases: lung and intestinal mucosa MCs discharge the neutral protease tryptase but not chymase, whereas skin and intestinal submucosal MCs release tryptase and chymase.

MCs have long regarded as key effector cells in the pathogenesis of allergic diseases. However, their ability to generate and release various cytokines points to their role in diverse pathophysiological processes such as wound healing, inflammatory reactions, angiogenesis, fibrosis and tumours (Gounaris *et al.* 2007; Ribatti 2015).

Nowadays, it is well known that MC granules contain preformed mediators that can be released after stimulation (Metcalf *et al.* 1997) and that MCs are important effector cells involved in host defense mechanisms and allergic processes. In fact, these cells play a key role in innate and acquired immunity where they contribute to inflammatory reactions through the release of different proinflammatory mediators such as histamine, heparin, lipid mediators or cytokines. During a type-I allergic reaction, immunoglobulin E (IgE) produced by B-lymphocytes binds to the membrane IgE receptor (the high affinity receptor for IgE, also referred to as FcεRI) on MC surface. When multivalent antigen

crosslinks the antigen-specific IgE bound to FcεRI receptors on MCs, preformed MC granule-associated vasoactive/proinflammatory mediators, including histamine, proteases and heparin are released immediately, while lipid mediators or cytokines are synthesized in a delayed manner. These mediators, and particularly histamine and lipid derivatives account for the successive adverse events associated with allergy such as increased vascular permeability, mucus secretion, and smooth muscle contraction occur (Metzger 1992; Kinet 1999; Siraganian 2003).

1.2. Development of mast cells

The origin of MCs was demonstrated for the first time in 1978 by Kitamura *et al.* who performed *in vivo* experiments using genetically MC-deficient mutant mice and suggested that MCs derive from bone marrow (BM) precursor cells (Kitamura *et al.* 1978). The hematopoietic origin of MCs in humans was later confirmed after allogeneic BM transplantation in a leukemic patient, where MCs isolated from the recipient's BM displayed the donor's genotype after 7 months of transplantation (Födinger *et al.* 1994). MCs develop from uncommitted hematopoietic stem cells that in turn give rise to committed CD34⁺ KIT⁺ CD13⁺ progenitor cells in the BM (Kirshenbaum *et al.* 1999). These progenitors are detectable in the peripheral blood (PB), BM, and umbilical cord blood (CB) (Ogawa *et al.* 1983; Valent *et al.* 1991; Valent *et al.* 1992; Mitsui *et al.* 1993; Arock *et al.* 1994; Valent 1995; Metcalfe *et al.* 1997) and express the receptor for stem cell factor (SCF) (SCF-receptor: CD117, or KIT) which binds to SCF, also termed KIT ligand (KL, also formerly termed steel factor (SF) and MC growth factor (MGF)). MC progenitors emigrate *via* the bloodstream into different tissues where they differentiate under the influence of various growth factors, including SCF (Abraham & Arock 1998; Metcalfe *et al.* 1997; Valent *et al.* 1991; Valent 1995; Ogawa *et al.* 1983; Valent *et al.* 1992; Mitsui *et al.* 1993; Saito *et al.* 1996; Galli *et al.* 1994; Arock *et al.* 1993; Wedemeyer *et al.* 2000). More specifically, a study showed that MCs are closely related to monocytic lineage since CD34⁺ KIT⁺ CD13⁺ cell population can give rise to both mature monocytes and MCs, hence *in vitro* promastocytes are preferentially described as CD34⁺ KIT⁺ CD13⁺ and negative for CD14, which is a lipopolysaccharide receptor specific for monocyte lineage (Kirshenbaum *et al.* 1999).

1.2.1. Stem cell factor and its effects on mast cell development

Stem cell factor (SCF) is produced mainly by stromal cells such as fibroblasts and endothelial cells. The mature protein SCF is either expressed on cell surface or released as a soluble growth factor. Fibroblasts and endothelial cells display the transmembrane form of SCF and also release the soluble form (Metcalf *et al.* 1997; Broudy 1997; Prussin & Metcalfe 2006). MCs are the only hematopoietic cells displaying a very high KIT expression during all stages of their development. Stem cell factor (SCF) has multiple biological effects on MCs, including modulating differentiation and homing, viability, MC hyperplasia and enhancement of mediator formation (Galli *et al.* 1995; Broudy 1997; Ashman 1999). Stem cell factor (SCF) can promote the survival of hematopoietic stem cells *in vitro* by increasing their entry into cell cycle (Broudy 1997). Moreover, MC progenitor stimulation by SCF leads to formation of granules and maturation of MCs. Recombinant human SCF (rhSCF) was reported to induce *in vitro* differentiation of human MCs from their BM and PB precursor cells in long-term culture and to upregulate MC releasability (Valent *et al.* 1992; Kirshenbaum *et al.* 1992). In addition, SCF has an important role in cell-matrix adhesion; it stimulates MCs to bind to fibronectin and, to a lesser extent, to vitronectin. In addition, the stimulation of integrin adhesiveness requires activation of the KIT protein tyrosine kinase (TK) (Kinashi & Springer 1994).

1.2.2. Effects of other cytokines and biological molecules on mast cell development in various species

In vitro generation of MCs requires absolutely SCF only for human promastocytes, but not for murine MC progenitors. Murine promastocytes develop into functional mucosal type MCs (MMCs) in culture in the presence of interleukin-3 (IL-3), and require SCF in addition to IL-3 only for the generation of connective type MCs (CTMCs) (Schrader 1981; Ihle *et al.* 1983). Apart from SCF and IL-3, several growth factors such as interleukin-4 (IL-4), interleukin-9 (IL-9), interleukin-10 (IL-10), and nerve growth factor (NGF) were found to regulate the development and differentiation of murine MCs (Matsuda *et al.* 1991; Valent *et al.* 1992; Rennick *et al.* 1995; Valent 1995; Metcalfe *et al.* 1997; Bischoff *et al.* 1999). In addition, IL-4 and IL-6 are known to promote the differentiation and/or

maturation of human MCs, whereas IL-9 and IL-10 has no such effect on the same cells (Valent *et al.* 1992; Galli *et al.* 1994; Valent 1995; Saito *et al.* 1996; Bischoff *et al.* 1999; Nakahata & Toru 2002). Moreover, IL-10 supports MC differentiation (Rennick *et al.* 1995) and may have anti-inflammatory effects on IgE/anti-IgE-challenged human MCs by inhibiting their release of IL-8, TNF- α and histamine (Royer *et al.* 2001; Kennedy Norton *et al.* 2008).

In addition, thrombopoietin (TPO), a hepatocyte-derived growth factor ligand for TPO receptor (CD110), was reported to enhance the development of a subpopulation of CD117^{low}/CD110+ human MCs derived from CD34+ pluripotent and CD34+/CD13+/CD117+ progenitor cells when used at specific concentration alone or in combination with SCF (Kirshenbaum *et al.* 2005, Metcalfe 2008).

Of note, lysophosphatidic acid (LPA) which is a phospholipid mediator produced and released by platelets, fibroblasts, and adipocytes upon phospholipase D-mediated reduction of lysophosphatidylcholine, was found to accelerate the development of human MCs in SCF-containing serum-free culture systems. LPA is comitogenic with SCF although it did not prolong MC survival (Bagga *et al.* 2004, Metcalfe 2008).

Finally, leukotriene D4 (LTD4), which is one of the cysteinyl (cys) leukotrienes (LTs) (cys-LT) and has the highest affinity for type 1 cys-LT G-protein-coupled receptor (CysLT1), was reported to increase the proliferation of human MCs derived from CB through mitogen-activated protein kinase/extracellular signal regulated kinase (MEK/ERK) and transactivation of the KIT receptor (Jiang *et al.* 2006, Metcalfe 2008).

Besides, MC development is also negatively regulated by other cytokines that inhibit their SCF-dependent differentiation from CD34+ progenitors. These negative regulators include interferon-gamma (IFN- γ) for human MCs (Kirshenbaum *et al.* 1998; Kulka & Metcalfe 2005; Metcalfe 2008), and transforming growth factor beta (TGF- β) for murine MCs (Kashyap *et al.* 1998). IFN- γ has an effect on early stages of MC development, while IL-5 inhibits MC development in later stages (Kulka & Metcalfe 2005; Metcalfe 2008).

Although IL-3 and IL-4 enhance human MC maturation, they inhibit MC development from their circulating progenitor cells in long-term culture (Sillaber *et al.* 1994; Xia *et al.* 1997; Oskeritzian *et al.*

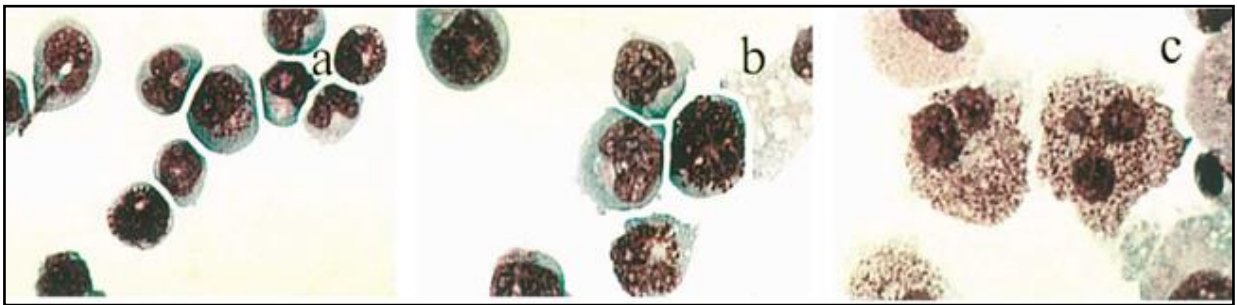


Figure 2: Morphology of cultured human MCs. CD34⁺ cord blood (CB) progenitor cells were cultured in SCF-containing medium and stained with Wright-Giemsa after the first two weeks (a,b) and at day 42 (c) (Figure adapted from Sperr *et al.* 2001).

1999; Kulka & Metcalfe 2005). Interleukin-4 decreases the number of MCs developing from fetal liver cells in SCF-containing medium and inhibits the expression of KIT and tryptase during SCF-dependent development of human MCs (Nilsson *et al.* 1994; Xia *et al.* 1997). Interleukin-3 (IL-3) is only active on the primitive MC-committed progenitors, where it promotes proliferation. However, IL-3 receptor alpha also known as CD123 is not any more expressed at later stages of MC development (Valent & Bettelheim 1992; Agis *et al.* 1996) and this interleukin is considered to recruit committed progenitors into non-MC lineage, resulting in a decreased MC development even in the presence of SCF in culture (Sillaber *et al.* 1994).

Regarding IL-4, its effects on human MC proliferation depend on the stage of maturation of MCs. Interleukin-4 may decrease the proliferation of MC-committed progenitors induced by SCF. That can be explained by the fact that IL-4 down-regulates KIT expression and enhances FcεRI expression (Sillaber *et al.* 1994; Toru *et al.* 1996; Oskeritzian *et al.* 1999; Kulka & Metcalfe 2005). However, several reports suggest that IL-4 may promote the proliferation of mature tissue MCs (Sillaber *et al.* 1994; Oskeritzian *et al.* 1999; Bischoff *et al.* 1999; Nakahata *et al.* 2002; Kulka & Metcalfe 2005; Metcalfe 2008).

1.3. Mast cell morphology, phenotype and heterogeneity

During the development and differentiation of human MCs, four morphologically distinct stages of maturation have been defined (Agis *et al.* 1996; Sperr *et al.* 2001; Horny *et al.* 2007). The most immature form of MCs is the non-granulated blast (Figure 2a-b), followed by metachromatically granulated blast. Mature MCs are well-granulated, ovoid or elongated irregularly cells with a round nucleus. Between non-granulated blasts and mature MCs, there is a promastocyte stage distinguished by its bi- or multi-lobed nuclei and generally hypogranulated cytoplasm. This cell is also termed atypical MC type II (Figure 2c) (Agis *et al.* 1996; Horny *et al.* 2007; Sperr *et al.* 2001). In all granulated stages, MCs are tryptase-positive.

In fact, human MCs express two main soluble tryptases that are secreted as tetramers during MC degranulation in response to different stimuli: α - and β -tryptases (β I, II, and III). Of these tryptases, β -

Table I: Distribution of human and murine MC proteases among MC subtypes. MCT, mast cell tryptase; MCTC, mast cell tryptase chymase; MMC, mucosal mast cell; CTMC, connective tissue mast cell; mMCP, mouse mast cell protease; MC-CPA, mast cell carboxypeptidase A (which is a Zn-dependent metalloprotease type, Pejler *et al.* 2007). MCTs have some characteristics of rodent MMCs whereas MCTCs have characteristics of rodent CTMCs (Table adapted from Vliagoftis & Befus 2005 and Pejler *et al.* 2010).

Species	MC subtype	Predominant localization	Tryptase	Chymase	MC-CPA
<u>Human</u>					
	MCT	Lung	Tryptase α		
			Tryptase β (I, II, III)		
	MCTC	Skin	Tryptase α	CMA1	MC-CPA
			Tryptase β (I, II, III)		
<u>Mouse</u>					
	MMC	Mucosal tissues		mMCP-1	
				mMCP-2	
	CTMC	Serosal surfaces	mMCP-6	mMCP-4	MC-CPA
			mMCP-7	mMCP-5	
				mMCP-9	

tryptase is the most biologically important as it does not have a catalytic domain defect which is responsible for the reduction of activity (Caughey 2007; Pejler *et al.* 2007; Metcalfe 2008; Pejler *et al.* 2010). The murine tryptases (mouse mast cell protease mMCP-6 and mMCP-7) are also tetrameric and most probably constitute the counterparts to human α - and β -tryptase. In addition, both human and mouse MCs express a monomeric, membrane-bound tryptase as well as a number of other trypsin-like serine proteases (Pejler *et al.* 2007; Caughey 2007; Stevens & Adachi 2007; Pejler *et al.* 2010).

In humans, tryptase is used as a specific immunohistochemical marker in order to detect and enumerate tissue MCs as this protease is present in all MC subtypes. Moreover, tryptase is of diagnostic importance because it can be measured in the serum and serves as an indicator of the total number of MCs. Baseline levels in healthy individuals is comprised between 0-15 ng/ml in healthy adult individuals (Horny *et al.* 2007).

The number of MC granules was reported to vary substantially between species and even in the same specie, and these organelles often display sub-structural patterns distinctive to the species (Dvorak 2005). The serine proteases including tryptases and chymases are the major protein components of MC secretory granules. The properties and the type of these proteases vary not only among species, but also among different MC subtypes (Pejler *et al.* 2007; Caughey 2007; Stevens & Adachi 2007; Metcalfe 2008; Pejler *et al.* 2010). Table I represents the distribution of human and murine MC proteases among different MC subtypes and the predominant localization of these subtypes. Two subtypes of MCs have been described in human tissues based on the proteases content of their granules and on their localization. MCs containing only tryptase (α and β), named MCT, are most abundant in mucosal tissues, whereas those containing tryptase together with chymase, carboxypeptidase and cathepsin G, called MCTC are mainly found in connective tissues (Irani *et al.* 1986; Schechter *et al.* 1990; Irani & Schwartz 1994; Vliagoftis & Befus 2005; Metcalfe 2008). These two subtypes are also heterogeneous with respect to their cytokine content, suggesting that they have different biologic functions as a result of their capacity to generate and release different cytokines. For instance, IL-5 and IL-6 seem to be expressed almost exclusively by MCT both in lung and in skin, whereas IL-4 is expressed primarily by MCTC (Bradding *et al.* 1995; Vliagoftis & Befus 2005). A

third, but minor, population of MCs which is found in lung and bowel and expresses chymase and cathepsin G, but not tryptase, and is thus termed MCC, has been reported and less studied (Weidner & Austen 1991; Metcalfe 2008). MCT subtype can be found mainly in the lung, the mucosa of the gut and the nose, whereas the MCTC subtype resides in the skin, tonsils and the submucosa of the gut (Irani *et al.* 1986; Schwartz *et al.* 1987; Irani & Schwartz 1994; Metcalfe *et al.* 1997; Vliagoftis & Befus 2005; Prussin & Metcalfe 2006). The microenvironment of the tissue in which MCs reside determines their sensitivity towards activating stimuli independently of their subtype. As an instance, skin MCs can be activated by poly-L-lysine, compound 48/80, the neuropeptide substance P, or morphine, whereas MCs of human lung, adenoids, tonsils, and colon are not responsive to these nonimmunologic stimuli (Lowman *et al.* 1988). In the mouse, MCs are divided into the connective tissue MC (CTMC) and mucosal MC (MMC) subtypes. CTMCs express predominantly two different chymases (the β -chymase mouse mast cell protease 4 (mMCP-4) and the α -chymase (mMCP-5), whereas MMCs express predominantly two types of β -chymases (mMCP-1 and mMCP-2). There are many distinguishing characteristics between these two MC populations. The granule core proteoglycan in CTMCs is primarily heparin (highly sulfated proteoglycan), whereas in MMCs, it is primarily chondroitin sulfate (lower level of sulfatation). There are also significant differences in morphology, histochemical characteristics, content of mediators, responsiveness to growth factors, sensitivity to drugs and stimuli, and biological functions (Arock *et al.* 1986; Schwartz *et al.* 1987; Welle 1997; Vliagoftis & Befus 2005; Rönnerberg *et al.* 2012). Culture of normal mouse hematopoietic cells for few weeks in presence of murine IL-3 develops into a heterogeneous MC population that resembles to mucosal MCs and clearly differ from serosal MCs (Arock *et al.* 1986).

MCs express a myriad of receptors on their surfaces. Among the most important receptors on MCs, two of them are KIT (CD117) and Fc ϵ RI (Ishizaka *et al.* 1979; Metzger 1992; Valent & Bettelheim 1992; Valent 1994; Okayama & Kawakami 2006; Kraft & Kinet 2007; Kim *et al.* 2008). Fc ϵ RI is only expressed on mature MCs, but not on immature MC progenitors, whereas KIT is always expressed on MCs regardless their maturation stage (Galli & Hammel 1994; Welker *et al.* 2000; Scherthaner *et al.* 2005; Valent *et al.* 2010). Furthermore, Fc ϵ RI is essential for the initiation of inflammatory-related

signaling, and KIT, the SCF receptor, plays an important role in MC development and homeostasis (Galli *et al.* 1993; Galli 2000; Okayama & Kawakami 2006; Kraft & Kinet 2007; Kim *et al.* 2008).

1.3.1. KIT, the tyrosine kinase receptor for stem cell factor (SCF)

As mentioned above, MCs and MC precursors express the receptor for SCF, namely KIT which is encoded by the *KIT* proto-oncogene (Valent 1994). The *KIT* proto-oncogene is identified as the cellular and untruncated counterpart of the gene in the Hardy-Zuckerman4 (HZ4) feline sarcoma virus genome (*v-Kit*) (Besmer *et al.* 1986; Yarden *et al.* 1987) and contains 21 exons transcribed/translated into a transmembrane tyrosine kinase receptor (TKR) that is structurally similar to the receptors for colony-stimulating factor-1 (CSF-1) and platelet derived growth factor (PDGF) (Chabot *et al.* 1988).

The KIT TKR is encoded at the white spotting (W) on chromosome 4q12 in human (Giebel *et al.* 1992) and on chromosome 5 in mouse (Yarden *et al.* 1987; Qiu *et al.* 1988). A number of mutations at the W locus have been documented (Russell 1979) and produce phenotypes ranging from embryonic lethality to severe macrocytic anemia, depigmentation, and sterility.

The protein KIT (CD117) has a molecular mass of 145 kDa with 976 amino acids in length (Giebel *et al.* 1992), and belongs to a family of transmembrane growth factor receptors with intrinsic TK activity (Qiu *et al.* 1988; Chabot *et al.* 1988). KIT is expressed at both RNA and protein levels in normal cell types including hematopoietic stem cells and progenitors (Andre *et al.* 1989; Simmons *et al.* 1994), melanocytes (Halaban *et al.* 1993), germ cells (Manova *et al.* 1990; Orr-Urtreger *et al.* 1990; Strohmeyer *et al.* 1995), interstitial cells of Cajal (Huizinga *et al.* 1995), glandular epithelial cells of breast (Lammie *et al.* 1994; Tsuura *et al.* 1994), normal MCs (Metcalf 2005), and melanocytes (Natali *et al.* 1992). The KIT protein is expressed not only in normal cells but also in neoplastic cells from gastrointestinal stromal tumours (GISTs) (Sarlomo-Rikala *et al.* 1998; Arber *et al.* 1998; Andersson *et al.* 2002; Went *et al.* 2004), small cell lung cancer (Sekido *et al.* 1991; Matsuda *et al.* 1993), colon cancer (Toyota *et al.* 1993), neuroblastoma (Beck *et al.* 1995), seminomas (Strohmeyer *et al.* 1995), breast cancer (Hines *et al.* 1995), MC disorders (Arber *et al.* 1998) and mastocytosis (Pardanani *et al.* 2006; Arock *et al.* 2015), acute myeloid leukemia (AML) (Bene *et al.* 1998), multiple

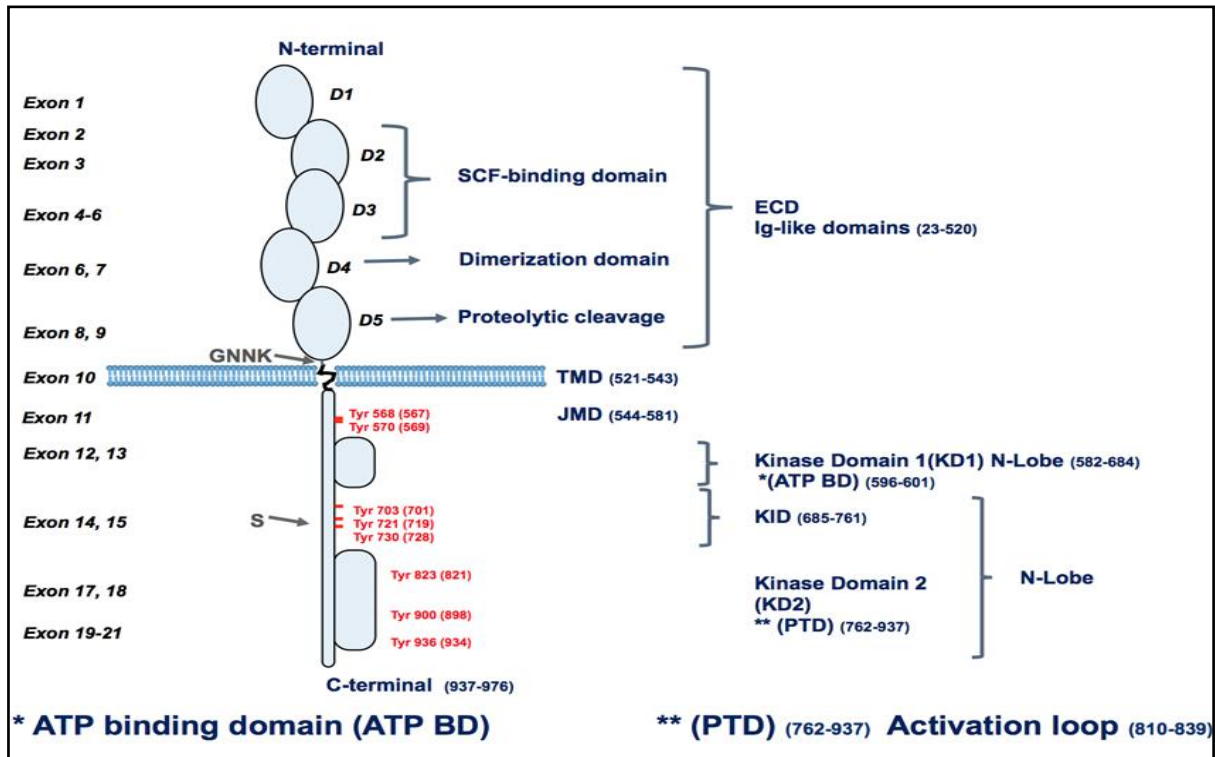


Figure 3: Schematic representation of the KIT structure and of its tyrosine phosphorylation sites. Phosphorylation of cytoplasmic tyrosine residues occurs via SCF binding. The human (mouse) numbering system is displayed. Two isoforms of Kit in the mouse and four in humans have been identified and result from alternate mRNA splicing (Ashman 1999). The four isoforms of human KIT which differ by the presence or absence of a tetrapeptide sequence, GNNK, in the extracellular region, or of an S residue in the interkinase region, as indicated. G refers to glycine; N, asparagine; K, lysine; S, serine. An additional splice variant resulting in the presence or absence of a single serine residue in the interkinase region of the cytoplasmic domain occurs in human KIT (Crosier *et al.* 1993) (Figure adapted from Ashman 1999, Roskoski 2005, Orfao 2007 and Bibi *et al.* 2014).

myeloma (MM) (Escribano *et al.* 1998), myelodysplastic syndromes (MDS) (Orfao *et al.* 2004), myeloproliferative disorders (MPDs) (Nakata *et al.* 1995), T-cell acute lymphoblastic leukemia (ALL) (Bene *et al.* 1998), B-cell non-Hodgkin lymphoma (Bravo *et al.* 2000), and B-cell precursor ALL (Bene *et al.* 1998). In normal cells, KIT has been reported to play a major role in gametogenesis (Kissel *et al.* 2000), melanogenesis (Nocka *et al.* 1989; Halaban *et al.* 1993), MC development and function (Valent *et al.* 2005; Metcalfe 2005), gastrointestinal function (Miettinen & Lasota 2005) and hematopoiesis in the differentiation of erythroid, lymphoid, megakaryocytic and myeloid precursors (Nocka *et al.* 1989).

The specific ligand of KIT, SCF, is primarily, but not exclusively, produced by stromal cells, such as fibroblasts, in two major forms; a soluble form and a membrane-bound form, as a result of differential splicing and proteolytic cleavage (Flanagan & Leder 1990; Toksoz *et al.* 1992 ; Broudy 1997; Ashman 1999; Reber *et al.* 2006). Both soluble and membrane-bound forms activate KIT. However, membrane-bound form induces more persistent TK activation and longer life span of *KIT* gene-encoded protein than the soluble form (Miyazawa *et al.* 1995; Ashman 1999).

Of note, KIT exists in active and inactive conformations as determined by X-ray crystallography and belongs to the type III receptor tyrosine kinase (RTK) subfamily, whose members include platelet-derived growth factor (PDGF) receptors α and β , macrophage colony-stimulating factor (M-CSF) receptor, and the Fms-like Tyrosine Kinase-3 (FLT3) receptor (Blume-Jensen & Hunter 2001). Its structure (Figure 3) consists of an extracellular domain (ECD), which contains the SCF-binding domain, a single transmembrane domain (TMD) encoded by exon 10, an autoinhibitory juxtamembrane domain (JMD) and a cytoplasmic kinase domain (KD) arranged in a proximal (N-) and a distal (C-) lobe linked by a hinge region and interrupted by a large kinase insert (kinase insertion domain (KID)) (Zhang *et al.* 2000; Roskoski 2005; Bibi *et al.* 2014). The ECD is encoded by the exons 1 to 9 and is characterized by its structure containing five immunoglobulin (Ig)-like domains (D1-5). SCF binds to the second and third immunoglobulin-like domains (D2 and D3) while the fourth domain (D4) plays a role in receptor dimerization (Chabot *et al.* 1988; Qiu *et al.* 1988; Zhang *et al.* 2000; Roskoski 2005).

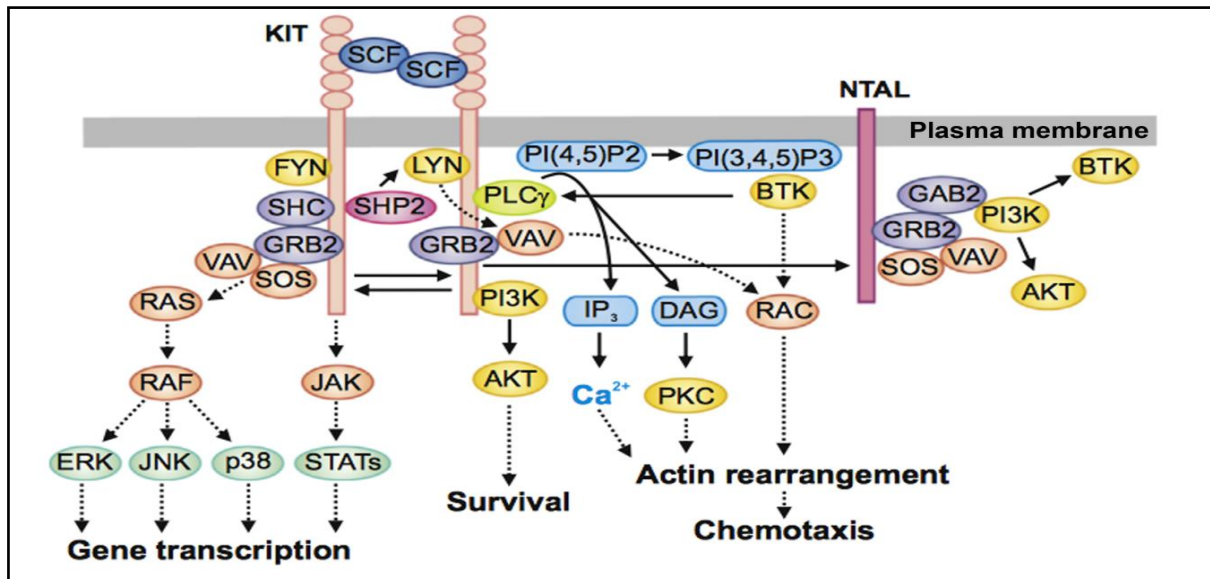


Figure 4: Schematic representation of signaling pathways downstream KIT. The KIT signaling occurs through several pathways. Stem-cell factor (SCF)-mediated dimerization of KIT leads to the receptor autophosphorylation at multiple tyrosine residues in the cytoplasmic tail, resulting in the recruitment of various molecules including cytosolic adaptor molecules, such as Shc (Src homology 2 (SH2)-domain-containing transforming protein C) and Grb2 (growth-factor-receptor-bound protein 2); Src family protein kinases (Lyn, Fyn); and signaling enzymes such as phospholipase C- γ (PLC- γ) and phosphatidylinositol 3-kinase (PI3-K). Activated PI3-K generates PI (3,4,5)P3 through which PH domain-containing proteins such as Bruton's tyrosine kinase (BTK) are recruited to the plasma membrane and further propagate the signal. Subsequent activation of these signaling enzymes, as well as the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway and the RAS-RAF-mitogen-activated protein kinase (MAPK) pathway leads to MC growth, differentiation, survival, chemotaxis and cytokine production. An increased activity of PLC- γ leads to production of DAG and IP₃, responsible for enhanced levels of free cytoplasmic Ca²⁺. This is followed by actin rearrangements and chemotactic response. KIT also phosphorylates NTAL that can bind other adapter proteins, including Grb2. Activated GRB2 orchestrates activation of RAS-RAF pathway by recruiting GEFs, the guanine nucleotide exchange factor Sos (son of sevenless homologue) and the guanine nucleotide exchange factor Vav, which subsequently leads to activation of MAP kinases ERK, JNK and p38. Some other signaling proteins such as JAK, STATs, AKT, PKC and Rac are involved in gene transcription, survival, actin rearrangement, and/or in chemotaxis (Figure adapted from Draber *et al.* 2015).

KIT dimerization by ligation of SCF is followed by conformational changes that enable lateral interactions between membrane proximal Ig-like domains D4 and D5 of two KIT molecules (Yuzawa *et al.* 2007). The proteolytic cleavage of the fifth immunoglobulin-like domain D5 of KIT from the cell surface converts the transmembrane protein into a soluble form (Broudy *et al.* 2001). The TMD forms a helix and is encoded by exon 10. The JMD encoded by exon 11 inhibits both KIT activity and receptor dimerization by its inserting into the kinase-active site between N- and C- lobes and stabilizing the inactivated conformation (Ma *et al.* 1999; Mol *et al.* 2003; Hubbard 2004; Mol *et al.* 2004; Roskoski 2005; Zou *et al.* 2008; Lemmon & Schlessinger 2010; Laine *et al.* 2012).

Binding of the protein-tyrosine phosphatase named SHP-1 (Src homology region 2 domain-containing phosphatase-1) to the phosphorylated KIT JMD results in dephosphorylation of the tyrosine residues and termination of the intracellular signal (Kozlowski *et al.* 1998). The structure of the class III receptors differs from that of other TKRs by the insertion of 70–100 amino acids near the middle of the kinase domain in the C-lobe. In human KIT, the kinase insert domain (KID), which is about 80 residues in length, undergoes phosphorylation and serves as a docking site for a few pivotal signal transduction proteins (Roskoski 2005).

1.3.2. KIT signaling pathways

KIT signaling occurs through several pathways including Src Family kinases pathway, PI3-kinase (PI3K)/AKT pathway, Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) (JAK/STAT) pathway, phospholipase C gamma (PLC- γ) pathway, and Ras/Raf/mitogen-activated protein kinases MAPKs pathway, leading to MC growth, differentiation, survival, adhesion cytokine production and chemotaxis (Roskoski 2005; Lennartsson *et al.* 2005; Gilfillan & Tkaczyk 2006; Bibi *et al.* 2014; Cruse *et al.* 2014; Draber *et al.* 2015) (Figure 4). In fact, the activation of KIT by SCF induces KIT dimerization and autophosphorylation by its intrinsic kinase activity of at least 10 tyrosine residues (Y568, Y570, Y703, Y721, Y730, Y747, Y823, Y900, Y917 and Y936, see Figure 3) that serve as docking sites for signal transduction molecules including cytosolic adapters Shc (Src homology 2 (SH2)-domain-containing transforming protein C), Gab2 (Grb2-associated binding

protein 2) and Grb2 (growth factor receptor bound protein 2), Src family PTKs (Lyn and Fyn), PLC- γ , and phosphatidylinositol 3-kinase (PI3-K) (Linnekin *et al.* 1997; Abram & Courtneidge 2000; Roskoski 2005; Lennartson *et al.* 2005). The early signaling event following the activation of KIT by SCF is the recruitment of Src family PTKs (Fyn and in particular Lyn) to phosphorylated tyrosine residues (Y568, Y570) in the JMD of KIT (Linnekin *et al.* 1997; Abram & Courtneidge 2000). Src family PTKs contain N-terminal myristoylation sites, and in some cases a palmitoylation domain, which are critical for anchoring the kinases to the plasma membrane and to the membrane microdomains (lipid rafts) (Kovarova *et al.* 2001). These events, which occur in the lipid rafts, are important for the signal transduction through PI3-K pathway because disruption of these lipid rafts specifically reduces the phosphorylation of PI3-K and the serine-threonine kinase AKT (also known as protein kinase B (PKB)) in response to SCF (Arcaro *et al.* 2007; Jahn *et al.* 2007). The PI3-K phosphorylates the plasma membrane-associated phosphatidylinositol-4,5-biphosphate (PI(4,5)P₂) to form phosphatidylinositol-3,4,5,-triphosphate (PI(3,4,5)P₃) (PIP₃), which in turn recruits pleckstrin homology (PH) domain-containing signaling proteins to the plasma membrane (Foster *et al.* 2003). The classic class I PI3-K complex consists of a regulatory p85 subunit and a catalytic subunit p110 (Munugalavadla *et al.* 2008). Activated KIT receptor can also directly bind the regulatory subunit of PI3-K (p85 α) and thus contributes to subsequent generation of membrane-associated PIP₃ (Roskoski 2005; Gilfillan & Rivera 2009). Phosphatidylinositol-3,4,5,-triphosphate (PIP₃) thereafter recruits and activates PH (Pleckstrin homology) domain-containing proteins such as Bruton's tyrosine kinase (BTK) and the serine-threonine kinase such as AKT (also known as protein kinase B (PKB)). BTK is recruited to the plasma membrane and further propagates the signal. Activated AKT in its turn phosphorylates Bad (Blume-Jensen *et al.* 1998), BIM (BCL-2 [B-cell lymphoma-2] interacting modulator of cell death) or the Forkhead transcription factor FOXO3a (forkhead box, class O3A) and downregulates the pro-apoptotic target BIM, leading to cell survival (Moller *et al.* 2005; Lennartsson *et al.* 2005). Phosphorylation of PI3-K activated by SCF contributes also to MC proliferation *via* the small GTPase Rac and c-Jun NH₂-terminal kinase (JNK), which activates the transcription factor c-Jun (Lennartsson *et al.* 2005).

The dimerization of KIT by SCF also triggers the activation of the MAPKs, extracellular signal regulated kinase 1 and 2 (ERK1 and ERK2), JNK, and p38. Phosphorylated tyrosine residues in activated KIT are recognized by the SH2 domain of the adaptor protein growth factor receptor-bound protein 2 (Grb2), which then forms a complex with the guanine exchange factor (GEF) son-of-sevenless (Sos). Sos activates the G protein Ras by promoting the exchange of GDP by GTP (Chardin *et al.* 1993). GTP-bound, active Ras initiates a cascade of serine/threonine kinases (Raf, MEK) that leads to the activation of the ERK1 and ERK2 (Roskoski 2012). The pathway leading to p38 and JUN amino-terminal kinase (JNK) activation by KIT in MCs is less well defined (Gilfillan & Tkaczyk 2006). The Ras-Raf-MEK-ERK pathway (Ras/Raf/mitogen-activated protein kinases MAPKs pathway) regulates many cellular processes, particularly survival, proliferation, and cytokine production in MCs. Indeed, activated Ras can interact with and initiate the activation of the serine/threonine kinase Raf-1. Active Raf-1 can then phosphorylate and activate the dual-specificity kinases Mek1 and 2. The mitogen-activated protein kinases Erk1 and Erk2 are serine/threonine kinases that are activated through phosphorylation by Mek1/2. Targets for Erks in the cytoplasm include the serine/threonine kinases RSK and Mnk1/2, as well as phospholipase A2 (PLA2). A fraction of the activated Erk and ribosomal S6 kinase (RSK) proteins translocate to the nucleus, where they phosphorylate various transcription factors, thereby influencing gene transcription (Lennartsson *et al.* 2005). Therefore, targeting this pathway is attractive for treatment of cancer (Montagut & Settleman 2009) and potentially of mastocytosis. The Ras-ERK pathway is activated by the interaction of the adaptor protein Grb2 with tyrosine residues 703 and 936 (Y703, Y936) of human KIT or indirectly *via* proteins that associate with KIT, such as Shc or Gab2 through SH2-domain-containing protein tyrosine phosphatase (SHP2).

The Janus kinases (JAKs) constitutively associate with KIT and become transiently activated in response to SCF, thereafter activated JAKs as well as activated KIT phosphorylate cytoplasmic STATs (STAT 1, 3, and 5) which then undergo a SH2 domain-dependent dimerization (Weiler *et al.* 1996; Deberry *et al.* 1997; Brizzi *et al.* 1999) and subsequent translocation to the nucleus. After SCF binding, STAT1, STAT5A, and STAT5B associate with KIT and become tyrosine phosphorylated

(Deberry *et al.* 1997; Brizzi *et al.* 1999). SCF-induced STAT tyrosine phosphorylation has been associated with increased DNA binding (Lennartsson *et al.* 2005). Nuclear STATs exert their functions by binding to their promoters and by regulating expression of responsive genes including *c-MYC*, *CIS*, *PIM-1* and *c-FOS* which play an important role in cell proliferation (Mui *et al.* 1996). STATs proteins (at least 1, 3, and 5) are also serine phosphorylated at the C-terminus, which regulates their transcriptional activity (Gotoh *et al.* 1996).

Interestingly, SCF, similarly to IgE-antigen complexes, can be an activator/co-activator of MCs (Gilfillan & Tkaczyk 2006). Indeed, when activated by antigen, SCF markedly increases MC degranulation and potentiates and prolongs calcium signals. It also influences MC chemotaxis and adhesion (Vosseller *et al.* 1997). The activation of KIT by SCF phosphorylates the adaptor protein non-T cell activation linker (NTAL) which in turn recruits and phosphorylates the phospholipase C-gamma1 (PLC- γ 1). This PLC- γ 1 is expressed ubiquitously whereas PLC- γ 2 is mainly expressed in hematopoietic cells (Wilde & Watson 2001). PLC- γ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG binds and activates the serine-threonine protein kinases PKCs, whereas IP3 induces release of Ca^{2+} from intracellular stores leading to cytokine production (Roskoski 2005). The PLC- γ association with KIT is dependent on tyrosine residues 730 and 936 of the human receptor (Herbst *et al.* 1995; Gommerman *et al.* 2000). However, soluble SCF has been reported to fail to increase the activity or phosphorylation of PLC- γ (Koike *et al.* 1993; Kozawa *et al.* 1997) suggesting activation of phospholipase D after SCF stimulation. In contrast, the membrane-bound form of SCF phosphorylates PLC- γ , suggesting its role in SCF-induced proliferation and survival (Gommerman *et al.* 2000; Trieselmann *et al.* 2003). The differential effects of soluble and membrane-bound SCF on signal transduction may be best explained by the fact that the membrane-bound SCF results in slower internalization of active KIT, whereas soluble SCF induces rapid internalization (Yee *et al.* 1994; Miyazawa *et al.* 1994; Miyazawa *et al.* 1995). Although it is well established that SCF protects KIT-expressing cells against apoptosis (Zsebo *et al.* 1992; Neta *et al.* 1993; Liebmann *et al.* 1994; Blume-Jensen *et al.* 1998), the biological effects of PLC- γ are less known. However, PLC- γ 1 has a critical role in SCF-induced protection against

apoptosis evoked by irradiation (Maddens *et al.* 2002) as well as by the cytotoxic agent daunorubicin (Plo *et al.* 2007).

Regarding KIT-mediated chemotaxis, SCF is known as a major chemotactic attractant for MCs and their precursors (Chabot *et al.* 1988; Meininger *et al.* 1992; Nilsson *et al.* 1994). Once bound to KIT, SCF causes KIT tyrosine phosphorylation and formation of docking sites for SH2 domain-containing molecules, such as Src family kinases Lyn and Fyn. This results in activation of the kinases and further propagation of the signal. Activation of Fyn leads to phosphorylation of Gab2 and subsequent activation of the small GTPase Rac that is responsible for the cytoskeletal reorganization and MC migration (Linnekin *et al.* 1997; Timokhina *et al.* 1998; Ueda *et al.* 2002; Samayawardhena *et al.* 2007). Phosphorylation of the tyrosine residue Y719 of KIT is crucial for recruitment of PI3-K, which plays an important role in MC chemotaxis towards different chemoattractants (Ueda *et al.* 2002). The protein tyrosine phosphatase PTP α is required for proper functioning of the Gab2/SHP2/Vav/RAC/JNK signaling axis, and reduced migration towards SCF has been reported in PTP α knockout mice (Samayawardhena & Pallen 2008). Chemotaxis towards SCF requires the phosphatase SHP2 which directly activates Lyn kinase by dephosphorylation of its inhibitory tyrosine, resulting in enhanced phosphorylation of Vav1, Rac activation and actin polymerization (Draber *et al.* 2015). Of note, BM derived MCs (BMMCs) from conditional SHP2 knockout mice exhibit decreased chemotaxis, and chemotaxis is also reduced in cells treated with a SHP2 inhibitor (Sharma *et al.* 2014).

1.3.3. Regulators of KIT kinase activity and KIT signaling

Since KIT activates multiple signaling pathways, its negative regulation also occurs through various mechanisms.

Receptor internalization, degradation and ubiquitination attenuate the strength and the duration of KIT signaling and thus have been considered as negative regulators of receptor signal transduction (Cruse *et al.* 2014). It is well known that ubiquitin molecules are covalently attached to lysine residues on target proteins, and this labels them for degradation *via* the 26S proteasome complex (Lennartsson *et*

al. 2005). Activation of KIT leads to its poly-ubiquitination (Miyazawa *et al.* 1994). The ubiquitin ligase Cbl has an important role in KIT ubiquitination and subsequent internalization, then degradation by the proteasome (Miyazawa *et al.* 1994) because it becomes phosphorylated in response to SCF through Src family kinases pathway and binds directly *via* its SH2 domain to Y568 and Y936 of activated KIT or through the adaptor protein Grb2 or tyrosine phosphorylated proteins CrkL, or the adapter proteins APS (Wisniewski *et al.* 1996; Sattler *et al.* 1997; Joazeiro *et al.* 1999; Wollberg *et al.* 2003; Sun *et al.* 2007).

Masson *et al.* have revealed insights into the mechanisms by which Cbl negatively regulates KIT-mediated signaling. Indeed, Cbl mediates monoubiquitination of KIT and the receptor is subsequently targeted for lysosomal degradation (Masson *et al.* 2006). Moreover, It has been shown that intact lipid rafts are required for the clathrin-dependent earliest stages of efficient internalization of KIT receptor and that this internalization depends on the recruitment of Src but not of PI3-K upon SCF stimulation using the induction of vesicular structures as a sign of efficient internalization of the receptor (Jahn *et al.* 2002). In fact, KIT can be internalized *via* the clathrin-coated pit mechanism and signal transduction by Src family kinases is not required for entry of KIT into clathrin-coated pits. In addition, KIT internalization is impaired after treatment with Src kinase inhibitor and also in Lyn-deficient cells, suggesting that Src family kinases, in particular Lyn, are involved in this process (Broudy *et al.* 1999). The PI3-K activity and Ca²⁺ influx also influence the late stages of KIT endocytosis and inhibition of these two signals prevents SCF-mediated internalization, leading to KIT degradation (Gommerman *et al.* 1997).

The protein kinases C (PKCs), which are serine-threonine kinases, can be activated through PI3-K and PLC pathways which generate DAG and intracellular Ca²⁺, the triggers of PKC activation. PKCs downregulate KIT in two ways. Indeed, PKC phosphorylates serine KIT residues 741 and 746 in the KID, resulting in the inhibition of KIT kinase activity (Blume-Jensen *et al.* 1994; Blume-Jensen *et al.* 1995). In addition, activated PKC also induces proteolytic release of the ECD of KIT (about 100 KDa) (Yee *et al.* 1993; Yee *et al.* 1994).

SHIP is a 5'-inositol phosphatase which hydrolyze phosphatidylinositol-3,4,5-trisphosphate (PIP3) into

PIP2. Interestingly, SHIP becomes phosphorylated upon SCF stimulation of MCs (Liu *et al.* 1997). Disruption of the *SHIP* gene has revealed that it is a negative regulator of SCF-induced MC degranulation (Huber *et al.* 1998). Furthermore, *SHIP*^{-/-} mice have granulocytes and macrophages with increased responsiveness to IL-3, GM-CSF, and SCF compared to wild type (WT) cells, supporting a role for SHIP as a negative regulator of cytokine signaling (Helgason *et al.* 1998). This tyrosine phosphatase SHIP negatively regulates KIT activity by interacting with its JMD (Tauchi *et al.* 1994; Kozlowski *et al.* 1998).

SHP1 (SH2 (Src homology-2) domain containing phosphatase 1) plays also a negative role when it is recruited to the tyrosine residue Y568 in the JMD of KIT and therefore dephosphorylates KIT directly or indirectly *via* other proteins associated with KIT (Kozlowski *et al.* 1998).

The suppressor of cytokine signaling SOCS-1 binds to KIT (Bayle *et al.* 2004) as well as to several downstream signal transduction proteins of KIT, including Grb2 and the guanine nucleotide exchange factor Vav and thereby inhibits JAK2 and SCF-mediated proliferation (De Sepulveda *et al.* 1999). SOCS-1 binds to Y567 in the JMD of KIT, ablating the activation of SCF-mediated MAPK pathway. SOCS-1 protein levels increase after SCF stimulation of MCs, counteracting the KIT-related induction of proliferation (De Sepulveda *et al.* 1999). BMNCs from SOCS-1 deficient mice have reduced proliferative response to both IL-3 and SCF, as compared with WT cells, due to accumulation of proteases in the absence of SOCS-1, resulting in degradation of essential signal transduction molecules. This data indicates that SOCS1 is required for the expression and/or stability of an endogenous protease inhibitor, which protects MCs from their own proteolytic enzymes (Ilanguvaran *et al.* 2003). SOCS-6 also negatively regulates KIT signaling (Bayle *et al.* 2004).

Another potential negative regulator of KIT is the adapter protein containing PH and SH2 domains APS. APS exists as a dimer and interacts with the JMD of KIT. This interaction facilitates the recruitment of Cbl, a negative regulator of TKs, into the complex (Yokouchi *et al.* 1999; Wollberg *et al.* 2003). Both KIT Y936 and Y570 seem to regulate the binding of APS and the recruitment of Cbl (Cruse *et al.* 2014). Lnk, a homologue of APS, is also tyrosine phosphorylated and regulates negatively KIT-mediated proliferation (Takaki *et al.* 2002). Dok-1 (downstream of tyrosine kinase 1)

is another potential negative regulator of KIT and may interact indirectly with the JMD (Liang *et al.* 2002).

The GTPase activating protein neurofibromin-1 (NF-1), which is a tumor suppressor commonly lost in juvenile myelomonocytic leukemia (JMML) (Aricò *et al.* 1997), has been suggested to be a negative regulator of KIT activity (Zhang *et al.* 1998). In *NF-1*^{-/-} progenitor cells, SCF increases colony formation and induces a stronger ERK activation as compared with normal cells (Lennartsson *et al.* 2005).

As described above, exon 11 of the cytosolic region of the JMD plays a critical role in the negative regulation of KIT dimerization. Indeed, residues 553-663 of exon 11 form a putative alpha helix that inhibits activation of ligand-unoccupied receptor (Ma *et al.* 1999; Mol *et al.* 2003; Mol *et al.* 2004). Mutations in the JMD of KIT play a critical role in regulating kinase activity by modulating the dimerization and potentially altering interaction with negative regulatory molecules including APS, Dok-1 and SHIP. Mutations in the JMD of KIT could alter the interaction with one or more of these negative regulators and thus result in cellular transformation (Lennartsson *et al.* 2005).

Mutations in the KD (PTD phosphotransferase domain) at codon 816 of KIT alter the conformation of the mobile activation loop (A-loop), weaken the binding of the JMD to the KD and suppress the negative autoinhibitory activity of the JMD on the KIT activity (Heinrich *et al.* 2002; Laine *et al.* 2011) as the tyrosine residues Y568 and Y570 in JMD are phosphorylated and thus their inhibition of KIT kinase is further disrupted (Blume & Hunter 2001; Laine *et al.* 2011; Lennartsson & Ronnstrand 2012). Structural studies of the active form of KIT WT suggest that Y823 of the A-loop is one of the last residues to be autophosphorylated (Mol *et al.* 2003). In addition, the recombinant KD with the D816Y mutation but not the WT KD was found to form dimers in solution (Kim *et al.* 2011), supporting the idea that extended JMD could make contact with another KIT receptor, thereby promoting dimerization in the absence of ligand. This further data led to the conclusion that the monomeric KIT KD is close to the active conformation but positioned in a way that prevents transactivation. Dimerization alters the conformation and orientation of the kinase domains allowing efficient autophosphorylation. This may explain the differences in substrate specificities of the JMD

mutants, which can dimerize like wild-type KIT, and of the kinase domain mutant that may dimerize through a different mechanism. Furthermore, this could also explain the difference in the sensitivity of these two mutants toward imatinib (STI571) (Lennartsson *et al.* 2005).

1.3.4. Gain-of-function mutations in KIT and mechanisms for constitutive activation

The oncogenic *KIT* mutations associated with human neoplasms are gain-of-function mutations that occur most frequently in the exon 11 encoding for the JMD or in the exon 17 encoding for KD. These gain-of-function mutations lead to SCF-independent TK activity and therefore result in ligand-independent proliferation, survival, and differentiation (Kitayama *et al.* 1996; Piao & Bernstein 1996; Ashman *et al.* 1999; Lennartsson & Ronnstrand 2012). Two gain-of-function mutations V560G and D816V were identified in the JMD and the second part of KD of KIT, respectively in the human MC line HMC-1 (Furistu *et al.* 1993). Since then, a variety of activating mutations in the JMD have been identified, including deletions, point mutations, tandem duplications, and combinations of deletions and point mutations. These mutations are frequently associated with GISTs (Duensing *et al.* 2004). Activating mutations in the second part of the KD have been identified as point mutations at codon 816 of human *KIT*, which corresponds to codon 814 in murine *Kit*. Aspartic acid 816/814 is located in the A-loop and can be mutated to tyrosine, histidine, asparagine, or valine with similar consequences (Furistu *et al.* 1993; Moriyama *et al.* 1996; Longely *et al.* 1996). Activating mutations in the KD of KIT are associated with human malignancies including mastocytosis as well as MC leukemia (MCL) (Nagata *et al.* 1995; Longely *et al.* 1996; Boissan *et al.* 2000; Garcia-Montero *et al.* 2006; Brockow & Metcalfe 2010; Bodemer *et al.* 2010; Verstoovsek 2013), germ cell tumors (D816H) (Tian *et al.* 1999), and less commonly melanoma (Tran & Tawbi 2012) and acute myeloid leukemia (AML) (Ashman *et al.* 1999; Malaise *et al.* 2009; Ashman & Griffith 2013). Other activating *KIT* mutations in the exon 8, 9 coding for ECD and exon 11 coding for TMD have been described in GISTs, childhood mastocytosis, and AML (Hirota *et al.* 1998; Kohl *et al.* 2005; Bodemer *et al.* 2010; Lennartsson & Ronnstrand 2012), but their incidence is much lower than that of mutations in exon 17 and 11 (Lennartsson & Ronnstrand 2012). These mutations in the ECD and TM of KIT increase its TK

activity by stabilizing the receptor dimers and facilitating its activation (Kohl *et al.* 2005; Yuzawa *et al.* 2007).

The activating mutations of KIT can be classified to either regulatory or enzymatic. The JMD mutations are considered regulatory since they likely change the conformation of KIT and lead to constitutive dimerization and activation by suppressing the auto-inhibitory effect of JMD on KIT activity, whereas mutations of the KD are catalytic and considered of enzymatic type (Kitayama *et al.* 1995; Longley *et al.* 2001; Laine *et al.* 2011).

Although both JMD and KD mutations of KIT result in its ligand-independent constitutive phosphorylation, the magnitude of phosphorylation generated by the KD mutants is generally greater than that of the JMD mutants (Hirota *et al.* 1998). The KD mutant *KIT* D816V in 293T cells has enhanced enzymatic activity when compared with WT KIT or with the JMD mutant *KIT* V559G (Furitsu *et al.* 1993; Lam *et al.* 1999; Chan *et al.* 2003). Moreover, the KD mutant (*Kit* D814V) exhibits an enhanced affinity for ATP, whereas the JMD deletion mutant and WT mutant bound ATP with similar affinity (Lam *et al.* 1999; Chan *et al.* 2003). Interestingly, these types of mutations differ in their sensitivity to inhibitors of KIT WT, including imatinib (STI571) and SU9529. Indeed, JMD mutants are sensitive to these inhibitors, in contrast to the KD mutants (Ma *et al.* 2002; Frost *et al.* 2002). However, even some JMD KIT mutants, such as V559I, do not respond to imatinib (Ashman & Griffith 2013).

In addition, a shift in substrate specificity has been observed with oncogenic KIT mutants. This phenomenon could contribute to differential activation of transforming pathways by mutant forms of KIT. KIT D816V was reported to exhibit a substrate specificity that resembled that of Src and Abelson murine leukemia viral oncogene homolog (ABL) tyrosine kinases. Therefore, this mutant KIT could activate ERK1/2 independently of Src whereas Src activity is essential for ERK1/2 in KIT WT (Sun *et al.* 2009). *In vitro* kinase assays using optimal peptide substrates for EGF receptor and ABL kinase showed a shift in substrate specificity with the D814Y mutation. Both WT and KIT D814Y phosphorylated an EGFR peptide, but KIT D814Y phosphorylated an ABL peptide much more efficiently (Piao *et al.* 1996). A similar shift in specificity was reported with a JMD deletion mutation

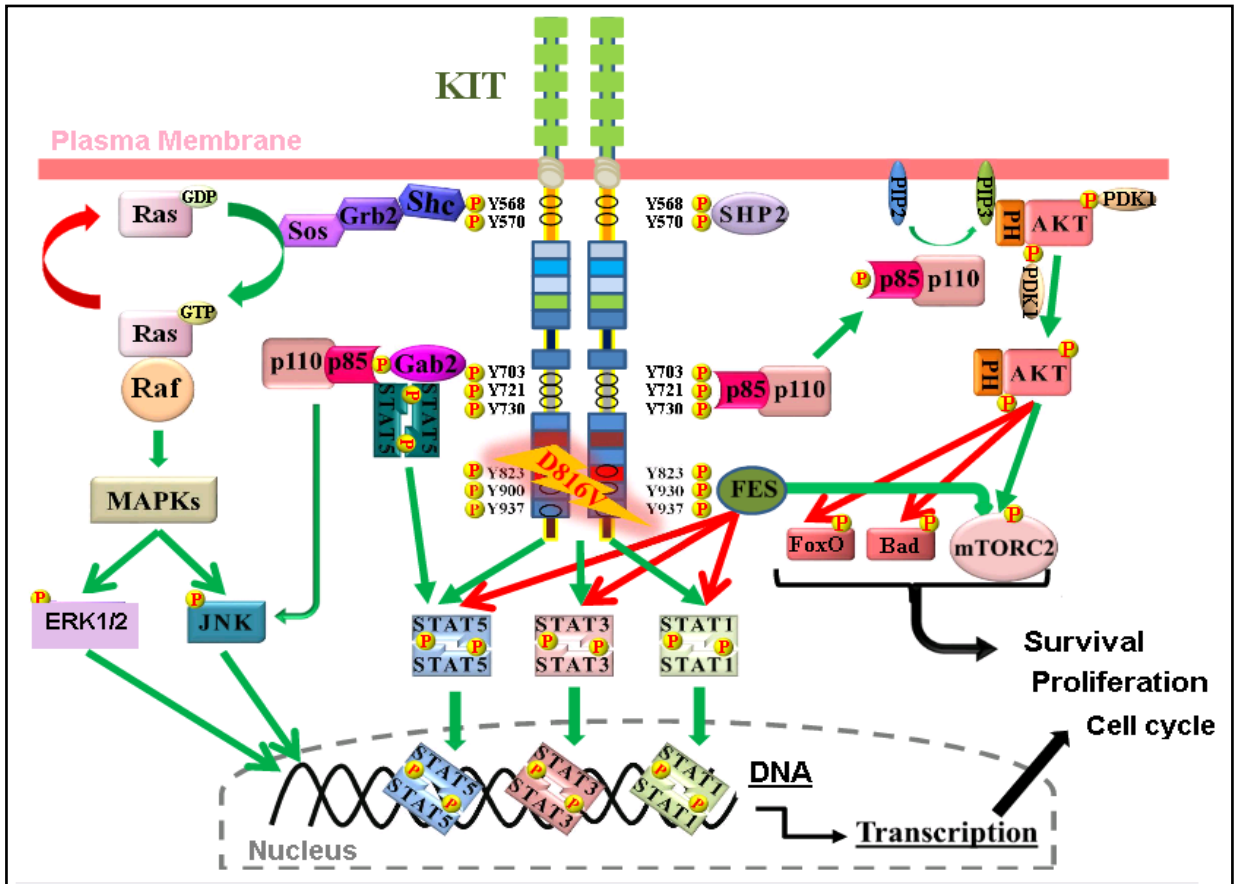


Figure 5: Aberrant signaling pathways downstream of KIT D816V mutant receptor. FES is permanently phosphorylated and negatively regulates (as indicated in red arrows) STAT pathways and activates (as indicated in red arrows) m-TOR pathway, resulting in induction of survival (black arrows) (Figure adapted from Bibi *et al.* 2014).

designated K Δ 27 at codons 547-555 compared with wild-type KIT (Casteran *et al.* 2003). Furthermore, feline sarcoma oncogene (FES) TK is activated by the D816V mutant and regulates negatively STAT pathway but induces mTOR phosphorylation (Voisset *et al.* 2007). Of note, the mTOR complex (mTORC1 and mTORC2) is upregulated in neoplastic immature MCs (Smrz *et al.* 2011). The mTORC1 may contribute to MC survival, whereas mTORC2 plays a crucial role only for homeostasis of neoplastic and dividing immature MCs, thus mTORC2 inhibition *via* a targeted approach may reduce the proliferation in MC disorders without affecting the normally differentiated MCs (Smrz *et al.* 2011). Interestingly, rapamycin, which inhibits mTOR, has been reported to decrease the growth and survival of *KIT* D816V positive MCs (Gabillot-Carré *et al.* 2006).

As previously underlined, the signal transduction induced by oncogenic mutants of KIT quantitatively and qualitatively differs from that induced by KIT WT. As an example, p38-MAPK pathways are preferentially activated over ERK-MAPK pathways in JM deletion mutants (Casteran *et al.* 2003). Similarly, the mitogenic pathways, such as AKT and STAT pathways are also constitutively activated in JM and D816V mutants (Chian *et al.* 2001; Ning *et al.* 2001; Casteran *et al.* 2003). Mutations in the ECD of KIT such as those found in some pediatric mastocytosis patients result in preferential AKT activation as compared to D816V mutations when introduced into rodent cells (Yang *et al.* 2010).

We have already described that KIT WT dimerizes and autophosphorylates after stimulation with SCF. JMD gain-of-function mutants form stable dimers in the absence of SCF. Similar to KIT WT, JMD mutants dimerize through the extracellular domain. However, it is unknown whether KIT D816V dimerizes spontaneously with KIT WT, or with itself, or is capable of transmitting oncogenic signals as a single molecule (Bibi *et al.* 2014). Nevertheless, it is well-known that the KIT D816V oncogenic mutation evokes aberrant signaling pathways of the receptor (Figure 5). Particularly, STAT5 has been reported to be involved in KIT D816V downstream signaling and thus in growth and survival of neoplastic MCs. Indeed, neoplastic MCs express both cytoplasmic and nuclear phospho-STAT5 and knockdown of *STAT5* in *KIT* mutant cells results in growth inhibition of neoplastic MCs (Harir *et al.* 2008; Baumgartner *et al.* 2009). AKT activation is also involved in KIT-dependent differentiation and growth of neoplastic MCs harboring oncogenic KIT mutants (Harir *et al.* 2008). Indeed, AKT was

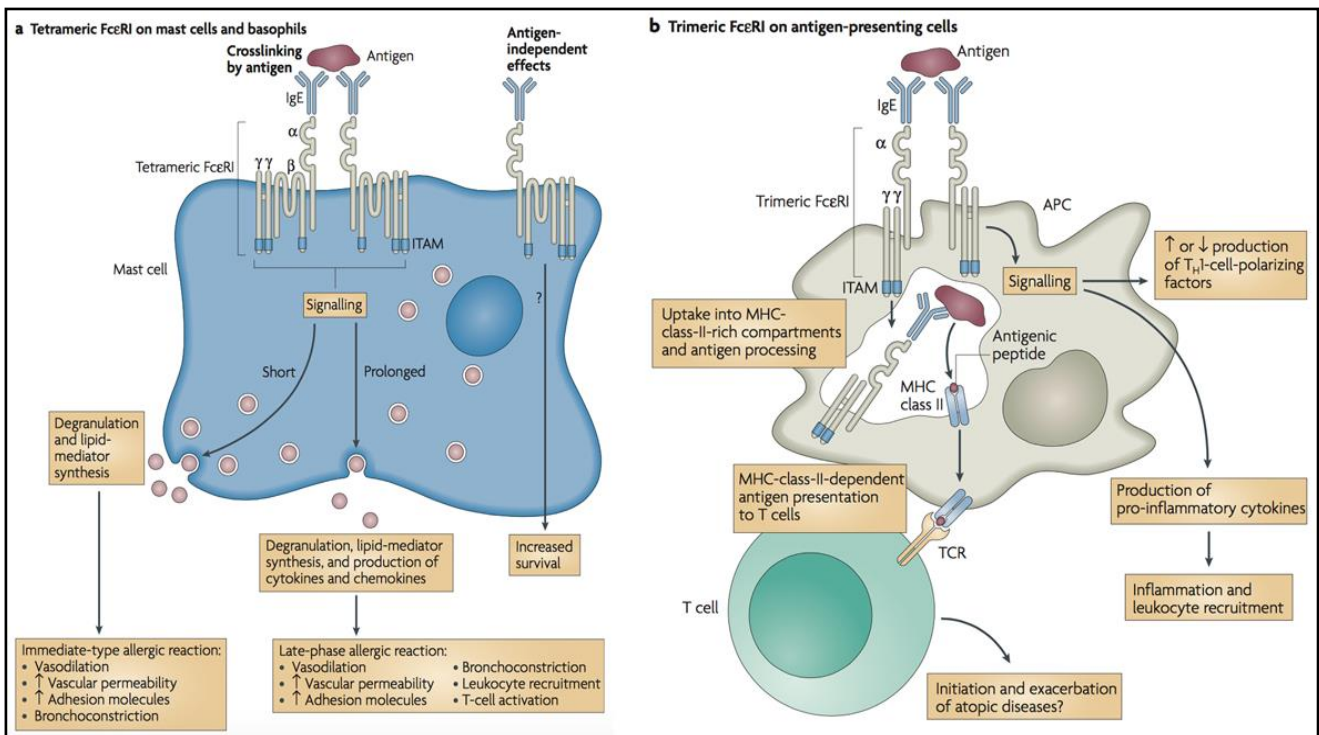


Figure 6: Composition and functions of complexes of FcεRI on mast cells (MCs) and basophils (Bas) as well as on antigen presenting cells (APCs). a. The tetrameric form of the FcεRI comprises the IgE-binding α chain with two immunoglobulin-like domains, the signal amplifying membrane-tetraspanning β chain and two γ chains. Both β and γ chains contain intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) whose phosphorylation of canonical tyrosine residues is essential for the signaling cascade. The tetramer form of FcεRI is expressed on MCs and Bas. b. The trimeric FcεRI lacks the β chain and is expressed by antigen-presenting cells (APCs) and eosinophils. It has influence on the uptake by major histocompatibility complex (MHC) class-II-rich compartments (Figure adapted from Kraft & Kinet 2007).

found phosphorylated in neoplastic MCs from patients with systemic mastocytosis (SM) harboring the *KIT* D816V mutant (Gabillet-Carré *et al.* 2006).

Furthermore, the Feline Sarcoma oncogene (FES) TK has been documented to be crucial for negative regulation of STAT pathway and for positive regulation of mTOR phosphorylation downstream of *KIT* D816V (Voisset *et al.* 2007). By contrast, this TK becomes activated following *KIT* WT stimulation *via* SCF but its phosphorylation is transient and results in a different final outcome: proliferation and survival are not dependent on FES while SCF-induced chemotaxis is (Voisset *et al.* 2010). Interestingly, FES is implicated in regulating the cross-talk between *KIT* and β 1-integrins to promote cytoskeletal reorganization and motility of murine MCs. In addition, SCF-induced chemotaxis of BMMCs was found defective in FES-deficient BMMCs, and restored in FES-rescued BMMCs (Smith *et al.* 2010).

1.4. The High affinity receptor for IgE (Fc ϵ RI)

1.4.1. Structure and expression of the Fc ϵ RI

The high affinity receptor for immunoglobulin E, designated Fc epsilon RI (Fc ϵ RI), is a member of multichain immune recognition receptor (MIRR) family responsible for linking pathogen- or allergen-specific IgE with cellular immunologic effector functions. This receptor binds the Fc fragment of IgE. The domain 3 of the constant region of heavy-chain of IgE is essential for the binding of the ligand to the receptor. In rodent, Fc ϵ RI is a tetramer ($\alpha\beta\gamma_2$), whereas it exists in trimeric ($\alpha\gamma_2$) as well as in tetrameric form ($\alpha\beta\gamma_2$) in human (Kinet 1999; Borkowski *et al.* 2001; Kraft & Kinet 2007; Blank *et al.* 2015). The structures and functions of both tetrameric and trimeric forms of Fc ϵ RI are presented in Figure 6. The cellular distribution of human and rodent Fc ϵ RI is also different. Rodent Fc ϵ RI is only expressed on MCs and basophils (Bas) (and non-B, non-T cells) (Kinet 1999), whereas in human the trimeric $\alpha\gamma_2$ Fc ϵ RI construct lacking the β -chain is expressed on Langerhans cells, eosinophils, platelets as well as on antigen presenting cells such as monocytes, macrophages, and dendritic cells (DCs) (Gounni *et al.* 1994; Maurer *et al.* 1994; Maurer *et al.* 1996; Joseph *et al.* 1997; Ying *et al.*

1998; Kinet 1999; Borkowski *et al.* 2001; Saini & MacGlashan 2002; Prussin & Metcalfe 2006; Knol 2006; Rivera *et al.* 2008). In humans, the tetrameric $\alpha\beta\gamma_2$ Fc ϵ RI complex is expressed only on Basophils (Bas) and MCs (Nadler 2000; Borkowski *et al.* 2001; Blank & Rivera 2004; Kraft & Kinet 2007; Rivera *et al.* 2008; Blank *et al.* 2015). Indeed, on the MC and Bas surfaces, a mixture of $\alpha\beta\gamma_2$ and $\alpha\gamma_2$ complexes is expressed, whereas only $\alpha\gamma_2$ structure is expressed on the antigen presenting cells (Turner & Kinet 1999). However, it has been recently reported that under certain conditions, including viral and plasmodium infection, rodent receptors can also be expressed in dendritic cells and neutrophils in the absence of β subunit (Grayson *et al.* 2007, Porcherie *et al.* 2011). The tetrameric complex includes a single IgE-binding α subunit, a single β subunit, and a disulfide-linked γ dimer whereas the trimeric structure comprises of one α subunit and γ dimer. The α -subunit, which binds IgE (Blank *et al.* 1991) comprises two extracellular immunoglobulin-like domains (D1 and D2), one transmembrane domain containing an aspartic residue, and a short cytoplasmic tail. The extracellular domain D2 contains two sites binding one of the two IgE heavy chains (Garman *et al.* 2000). The α -chain is heavily glycosylated since it contains seven N-linked glycosylation sites. These N-linked oligosaccharides are required in the endoplasmic reticulum (ER) to mediate the proper interaction between the α -chain and the ER folding machinery (Letourneur *et al.* 1995; Kinet 1999). Thus, the cytoplasmic part of α -chain has no role in downstream signaling while the β and γ chains contain unique cytoplasmic domains essential for downstream signaling, called immunoreceptor tyrosine based activation motifs (ITAMs). In fact, the aggregation of IgE-bound Fc ϵ RI induced by specific multivalent antigen (Ag) induces the phosphorylation of tyrosine residues in these motifs, which initiates in turn an intracellular signal transduction cascade and, as a consequence, the release of mediators of the allergic response (Benhamou *et al.* 1990; Kinet 1999; Knol 2006; Kraft & Kinet 2007). The sequence of the ITAMs differs between the two chains. The γ -chain ITAM sequence is (Asp/Glu)-X-X-Tyr-X-X-Leu-(X)-Tyr-X-X-Leu/Ile), where X stands for any amino acid. The β -chain has a slightly different sequence with a third tyrosine between the canonical tyrosine residues and the short spacer region. The tyrosine residues represent phosphor-acceptor sites that are important for the course of receptor-associated protein PTKs. The ITAMs further comprise the docking sites for

cytoplasmic proteins containing the Src-homology 2 (SH2) domain which are the linkage between receptor and the following signal transduction cascade (Knol 2006; Kraft & Kinet 2007). The 33 kDa signal-amplifying β chain consists of 243 amino acids and has four transmembrane domains, two extracellular loops and a single cytoplasmic loop, separating amino and carboxy terminal cytoplasmic tails (Kinet 1999; Knol 2006). The β subunit (Fc ϵ RI β) amplifies the signal strength and enhances the cell surface expression of unoccupied receptors (Knol 2006; Kraft & Kinet 2007; Borkowski *et al.* 2001). Furthermore, the Fc ϵ RI β functions to amplify responses under a low-intensity stimulus, whereas at a high intensity of stimulus, it might exert a negative regulatory role (Xiao *et al.* 2005). The γ -chains have a transmembrane structure consisting of five amino acid extracellular domains, a 21 amino acid transmembrane component and a cytoplasmic part of 36 amino acids. The γ -chains represent the major Fc ϵ RI signaling units of the Fc ϵ RI (Knol 2006; Kraft & Kinet 2007). Interestingly, both the β - and γ -chains are shared with other Fc receptors. For example, the high and low affinity receptor for IgG (Fc γ RI and Fc γ RIII, respectively) and the Fc receptors for IgA (Fc α RI) associate with the γ -chain depending on the cellular context (Knol 2006; Kraft & Kinet 2007).

1.4.2. Regulation of Fc ϵ RI expression

Cell surface expression of Fc ϵ RI is regulated by the concentration of IgE (Borkowski *et al.* 2001; Kraft & Kinet 2007). A relationship between the concentration of IgE in serum and cell-bound IgE exists and was first observed in human Bas in the PB (Malveaux *et al.* 1978; Borkowski *et al.* 2001; Kawakami & Galli 2002; Kraft & Kinet 2007).

IgE monomer binds the Fc ϵ RI α -chain with high affinity and this binding strongly upregulates the expression of Fc ϵ RI (Kawakami & Galli 2002; Mirkina *et al.* 2007; Kraft & Kinet 2007), by a mechanism which involves stabilization of the receptor at the cell surface by preventing receptor internalization and degradation, by recruiting a preformed pool of receptors comprising recycled and recently synthesized receptors and by stimulating continued basal level of protein synthesis (Borkowski *et al.* 2001). In addition, low Fc ϵ RI expression levels on MCs from IgE-deficient mice could be significantly increased by *in vitro* incubation with or by *in vivo* injection of IgE (Yamaguchi

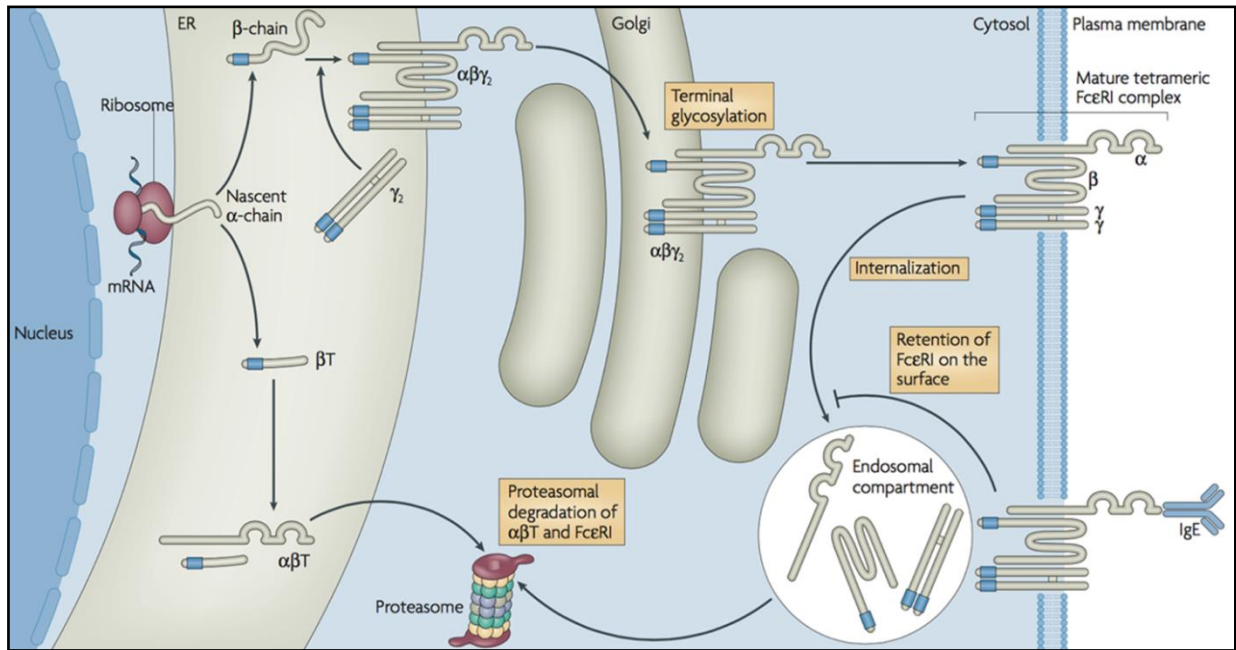


Figure 7: Regulation of surface expression of Fc ϵ RI on human mast cells (MCs) and basophils (Bas). Interleukin-4 (IL-4) promotes the production of immature core-glycosylated α -chains of Fc ϵ RI in the endoplasmic reticulum (ER). These immature chains remain in ER in the absence of β - and two γ - chains, while they are exported with β and γ chains after blocking of their retention signals from ER to the cell surface. However, this process happens after the full maturation of the Fc ϵ RI by terminal glycosylation in the Golgi compartment, and is more efficient for the tetrameric form of Fc ϵ RI which is more stable on the cell surface than the trimeric Fc ϵ RI lacking the β -chain. IgE, the Fc ϵ RI ligand, stabilizes Fc ϵ RI complexes on the cell surface and blocks their internalization. The inhibition of Fc ϵ RI internalization in turn leads to the accumulation of more Fc ϵ RI complexes on the cell surface because of the ongoing Fc ϵ RI synthesis. However, the truncated β chain variant (β T) produced by alternative splicing prevents the Fc ϵ RI complex from reaching the cell surface and results in proteasomal degradation of immature Fc ϵ RI complexes (Figure adapted from Kraft & Kinoshita 2007).

et al. 1997; Kawakamai & Galli 2002; Kraft & Kinet 2007). Furthermore, it has been described that IgE bound to their receptors will rest permanently at the cell surface whereas empty receptors are expressed only for some hours (MacGlashan 2007; MacGlashan 2008). Figure 7 shows the regulation of cell surface expression of FcεRI on human MCs and Bas.

1.4.3. Signal transduction downstream FcεRI

Although FcεRI has no intrinsic TK activity, the interaction between the antigen (Ag) and the receptor-bound IgE induces the activation of various signaling pathways (Kawakami & Galli 2002; Rivera *et al.* 2008). In the last few years, two pathways of the FcεRI signaling cascade were described and analyzed, a primary pathway and a complementary pathway regulating the FcεRI-mediated degranulation (Kraft & Kinet 2007). To explain the molecular mechanism of the initial steps of FcεRI signaling, including the phosphorylation of the receptor, three models have been proposed: a transphosphorylation model, a lipid raft model, and a PTK-protein tyrosine phosphatase (PTP) interaction model (Bugajev *et al.* 2010).

The activation of PTKs such as Src, Syk and Tec family is induced through the crosslinking of the FcεRI by specific IgE and the corresponding antigen, and caused by phosphate transfers (Kawakami *et al.* 2000; Kraft & Kinet 2007; Rivera *et al.* 2008; MacGlashan 2008). After the aggregation of the receptor, phosphorylation of the tyrosine residues of the ITAMs of the β and the γ chains occurs. This phosphorylation is often caused through transphosphorylation by Lyn, a nonreceptor Src family PTK which is mainly associated with the β subunit of the receptor (Kawakami & Galli 2002; Knol 2006; Kraft & Kinet 2007; Rivera *et al.* 2008; Gilfillan & Rivera 2009). Lyn kinase activity is the key for phosphorylation of the tyrosine residues in ITAM motifs through transphosphorylation (Pribluda *et al.* 1994). The phosphorylated ITAMs of the β and the γ chain are scaffolds for the binding of the cytoplasmic signaling molecules with SH2 domains like additional Lyn and Syk as well as Fyn (another src family PTK). While Lyn and Fyn are associated with the β chain, Syk is associated with the γ chain (Kawakami & Galli 2002; Knol 2006; Kraft & Kinet 2007; Rivera *et al.* 2008; Gilfillan & Rivera 2009; Alvarez-Errico *et al.* 2009). Fyn is also known to associate with FcεRI and to play a

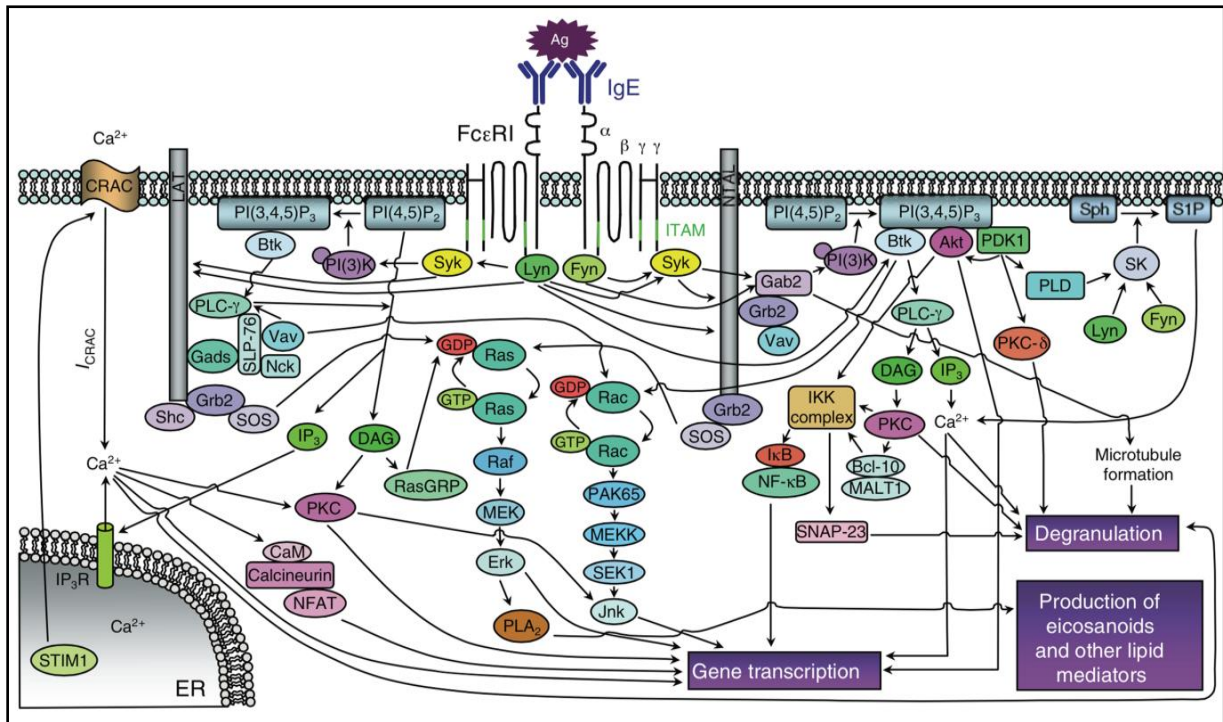


Figure 8: Intracellular signaling and cellular events following aggregation of FcεRI in MCs (Figure from Kalesnikoff & Galli 2008).

complementary role by activating PI3-K (Parravicini *et al.* 2002). However, unlike Lyn, which can be found primarily in lipid rafts, only a small amount of Fyn seems to reside in these domains (Kovarova *et al.* 2006). Through its interaction with the phosphorylated γ chain, Syk adopts an active conformation, leading to its phosphorylation by Lyn and subsequently transphosphorylation of other Syk molecules, which amplifies Syk activation and allows downstream phosphorylation of its targets (Eiseman & Bolen 1992; Hutchcroft *et al.* 1992; Jouvin *et al.* 1994; Parravicini *et al.* 2002; Kawakami & Galli 2002; Siraganian 2003; Kraft & Kinet 2007). Then, active Syk phosphorylates many substrates/proteins such as the adaptor protein linkers for activation of T cells (LAT) (Saitoh *et al.* 2000), non-T cell activation linker (NTAL) also known as linker for activated B cell (LAB) (Zhu *et al.* 2004), SH2-containing leukocyte-specific protein of 76 kDa (SLP76) (Hendricks-Taylor *et al.* 1997), Vav (Teramoto *et al.* 1997), phospholipase C-gamma (PLC- γ : PLC- γ 1 and PLC- γ 2). In MCs, PLC- γ 2 is absolutely required for Fc ϵ RI-mediated calcium mobilization, degranulation, and cytokine production, which cannot be compensated by the concurrently expressed PLC- γ 1 (Wang *et al.* 2000). Whatsoever, these events further lead to the activation of several signaling cascades including PI3-K, RAS/ERK, c-Jun N-terminal kinase (JNK), and BTK (Kawakami & Galli 2002; Siraganian 2003; Kraft & Kinet 2007; Rivera *et al.* 2008; Kalesnikoff & Galli 2008; Siraganian *et al.* 2010) (Figure 8). PLC- γ 1 and PLC- γ 2 belong to PLC family and catalyze the hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG), leading to the activation of protein kinase C (PKC) and the mobilization of intracellular Ca²⁺ from the endoplasmic reticulum (ER). The release of Ca²⁺ from the ER leads to stromal interaction molecule 1 (STIM1)-mediated opening of the store-operated Ca²⁺ channel ORAI1, which leads to the influx of extracellular Ca²⁺. The influx of Ca²⁺ is amplified by an additional mechanism which is mediated by short transient potential Ca²⁺ channel 1 (TRPC1). Indeed, the depletion of ER Ca²⁺ stores results in Ca²⁺ influx across the plasma membrane by store-operated calcium release-activated calcium (CRAC) channels in the plasma membrane. ORAI1 is the pore subunit of the CRAC channel while STIM1 is ER Ca²⁺ sensor. The depletion of calcium in the ER induces STIM1 formation of multimers in the ER membrane which move to sites of ER plasma

membrane apposition and interact directly with ORAI channels which leads to the influx of extracellular Ca^{2+} and a calcium release-activated current (ICRAC). Further the increase in intracellular Ca^{2+} , with the participation of membrane fusion proteins called SNAREs (soluble N-ethyl-maleimide-sensitive factor [NSF] attachment protein receptors), allows granular fusion and release of their content (Blank & Rivera 2004; Kalesnikoff & Galli 2008; Siraganian *et al.* 2010). The increase in intracellular Ca^{2+} levels and the activation of PKC are thus essential triggers of FcεRI-regulated degranulation (Turner & Kinet 1999; Siraganian 2003; Knol 2006; Blank & Rivera 2004). Thus, granules translocate from the cell interior towards the plasma membrane in a microtubule-dependent manner. Moreover, Phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) is an important lipid mediator that regulates the activity of various enzymes, such as BTK, the serine-threonine kinase AKT, the phosphoinositide-dependent kinase PDK1, phospholipase D (PLD) (Hitomi *et al.* 2004) and sphingosine kinase (SK), and the formation of other lipid mediators, such as diacylglycerol (DAG) and sphingosine 1-phosphate (S1P). S1P can act intracellularly to regulate Ca^{2+} influx leading to degranulation independently of PLC and IP3 or extracellularly after secretion from the cell by binding to the S1P1 or S1P2 surface receptor, thereby inducing cytoskeletal rearrangement or enhancing degranulation respectively (Kalesnikoff & Galli 2008).

An additional pathway results in the Fyn-dependent tyrosine phosphorylation of the Gab2, which further binds the p85 subunit of PI3-K. This step results in the activation of the phosphoinositide-dependent kinase 1 (PDK1) and subsequently in the initiation of the protein kinase Cδ (PKCδ) which further regulates degranulation. Moreover, the activation of PKCδ can cause activation of AKT, resulting in anti-apoptotic effects as well as starting cytokine production (Parravicini *et al.* 2002; Siraganian 2003; Kraft & Kinet 2007; Rivera *et al.* 2008).

The increase in intracellular Ca^{2+} concentration also activates the Ca^{2+} /calmodulin-dependent serine phosphatase calcineurin. Calcineurin dephosphorylates the nuclear factor for T cell activation (NFAT) which exposes NFAT nuclear-localization signals. Once in the nucleus, NFAT regulates the transcription of several cytokine genes. Transcriptions of other proinflammatory mediators are regulated by NFκB proteins which are transcription factors retained in the cytoplasm by binding to the

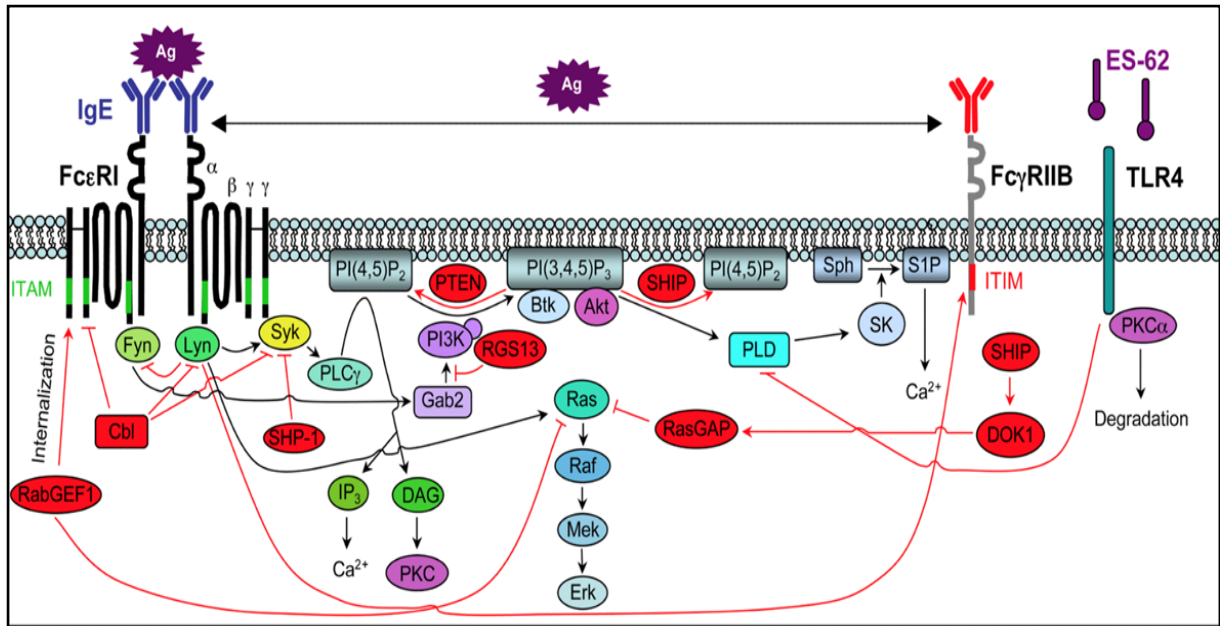


Figure 9: The negative regulation of FcεRI-mediated signaling events (Figure from Kalesnikoff & Galli 2008).

inhibitor I κ B. Fc ϵ RI stimulation leads to phosphorylation, thus degradation of I κ B, which allows the release and nuclear translocation of the NF κ B proteins. Protein kinase C β (PKC β), B cell lymphoma 10 (BCL10), and mucosa-associated-lymphoid-tissue lymphoma-translocation protein 10 (MALT10) are other proteins involved in this pathway (Ruland *et al.* 2003). Activation of the PI3-K and mitogen-activated protein (MAP) kinases and of the NFAT and NF κ B transcription factors then promotes transcription factors for the production of several cytokines. Another substrate of PKC and the MAP kinases is cytoplasmic phospholipase A2 (PLA2) which releases arachidonic acid that is metabolized by the cyclooxygenase or the lipoxygenase pathways to form the inflammatory mediators, prostaglandins and leukotrienes.

To sum up, each of the Lyn and the Fyn pathways is crucial for specific signaling events. The Lyn pathway plays an important role in the Ca²⁺ signaling, whereas the Fyn pathway is essential for the degranulation and the maintenance and/or the amplification of the Ca²⁺ signal. In Fyn-deficient mice, PI3-K and degranulation are affected, but only little effects can be observed on Ca²⁺ signaling. By contrast, the Ca²⁺ signaling is inhibited and degranulation is enhanced in Lyn deficient mice, suggesting that the Lyn pathway is a negative regulator of the Fyn pathway (Kraft & Kinet 2007). An important fact is that both the Lyn and the Fyn pathways are dependent on the Syk pathway because the Ca²⁺ mobilization, as well as the degranulation, are diminished in Syk-deficient mice. This hypothesis could also be supported in Syk-deficient non-releaser Bas, where through incubation with IL-3 and the presence of Syk mRNA, Syk protein expression and the Fc ϵ RI-dependent secretion could be re-established (Kepley *et al.* 2000; Sanderson *et al.* 2010).

Fc ϵ RI aggregation also activates and recruits negative regulators which limit the intensity of the positive signals activated downstream of this receptor (Figure 9). In addition to its role in initiating the activating signals as mentioned above, Lyn also negatively regulates Fc ϵ RI-induced signaling events by activating negative regulators (Kawakami *et al.* 2000; Odom *et al.* 2004; Hernandez-Hansen *et al.* 2004; Xiao *et al.* 2005). Several other negative regulators include for c-Cbl proteins which, by promoting ubiquitination of the activated receptor subunits and associated protein tyrosine kinases, decrease their levels (Rao *et al.* 2001; Kalesnikoff & Galli 2008; Gasparrini *et al.* 2011), the lipid

phosphatases including SHIP (Src homology 2 (SH2) domain-containing inositol 5'-phosphatases, SHIP-1 and -2) (Huber *et al.* 1998; Kalesnikoff *et al.* 2002; Kalesnikoff *et al.* 2003; Hernandez-Hansen *et al.* 2004; Xiao *et al.* 2005; Gimborn *et al.* 2005; Leung & Bolland 2007; Molfetta *et al.* 2007) and PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Furumoto *et al.* 2006; Molfetta *et al.* 2007) both of which hydrolyze PIP3. Amongst the negative regulators, the following have also been described: the tyrosine phosphatases SHP1 (SH2 domain-containing protein tyrosine phosphatase) that can dephosphorylate Syk (Odom *et al.* 2004; Molfetta *et al.* 2007), the linker for activation of X cells (LAX where X denotes an as-yet-unidentified cell) (Zhu *et al.* 2006) which binds Grb2 and the p85 subunit of PIK3, the exchange factor RabGEF1 (Rab5 guanine nucleotide exchange factor) which controls FcεRI internalization and can bind to GTP-bound Ras and negatively regulates Ras/Raf1/ERK pathways (Tam *et al.* 2004; Molfetta *et al.* 2007; Kalesnikoff & Galli 2008). The downstream tyrosine kinase (Dok1) which upon activation binds RasGAP; the exchange factor enhancing the intrinsic GTPase activity of Ras; and thus negatively regulates Ras/Raf1/ERK pathways and TNF-α production (Abramson *et al.* 2003; Molfetta *et al.* 2007; Kalesnikoff & Galli 2008), and RGS13 (regulator of G protein signaling (RGS) 13 which binds to the p85α subunit of PI3K and disrupts its association with Gab2 and Grb2 (Bansal *et al.* 2008; Kalesnikoff & Galli 2008).

Another important fact is that MCs are able to recover and to regranulate after IgE-mediated activation *via* FcεRI, especially in allergic responses (Dvorak 2005; Xiang *et al.* 2006). Once bound to FcεRI on resting MCs, IgE can persist on the cell surface for long time periods. IgE does not only interact with the FcεRI, but also with the IgG receptors FcγRII and FcγRIII as well as with galectin-3 (Kawakami & Galli 2002).

Other negative signals are generated by recruitment of negative signaling molecules such as SHIP or SHP to immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing receptors which promote dephosphorylation reactions and mechanistically suppress IgE-mediated cell activation (Katz 2002; Bruhns *et al.* 2005; Daëron *et al.* 2008). These ITIM-bearing receptors include the low affinity Fc receptor for IgG (FcγRIIB also known as CD32) (Ono *et al.* 1996; Malbec *et al.* 1998; Ott *et al.* 2002;

Katz 2002; Siraganian 2003; Tam *et al.* 2004), Platelet endothelial cell adhesion molecule-1 PECAM-1 (also known as CD31) (Wong *et al.* 2002), mast-cell-function-associated antigen (MAFA) (Ortega *et al.* 1991; Guthmann *et al.* 1995; Guthmann *et al.* 1995; Jürgens *et al.* 1996; Xu *et al.* 2001; Abramson *et al.* 2002; Abramson *et al.* 2006), the glycoprotein 49B1 (GP49B1) (Katz *et al.* 1996; Lu-Kuo *et al.* 1999; Katz 2002), the leukocyte immunoglobulin-like receptors (LIRs) (Bellón *et al.* 2002), the paired Ig-like receptor B (PIR-B) (Maeda *et al.* 1998; Yamashita *et al.* 1998; Uehara *et al.* 2001), the signal regulator protein α (SIRP α) (Liénard *et al.* 1999), the myeloid-associated immunoglobulin-like receptor (MAIR-1), also called CD300a (Yotsumoto *et al.* 2003; Kumagai *et al.* 2003; Bachelet *et al.* 2005), and the allergy inhibitor receptor 1 (Allergin1) (Katz 2002; Hitomi *et al.* 2010).

During the activation steps by Lyn, the tyrosine residues in ITIMs are phosphorylated and then recruit inhibitory effectors such as SHIP, SHP-1 or SHP-2. In some but not all cases, the negative signals are generated by Ag-induced co-aggregation of Fc ϵ RI with negative receptors. A chimeric fusion protein containing part of the Fc portion of IgG and IgE crosslinks Fc ϵ RI to Fc γ RIIB, and inhibits antigen-induced Syk phosphorylation and the degranulation of human Bas (Zhu *et al.* 2002). Finally, ES-62 the glycoprotein secreted by filarial nematodes forms a complex with toll like receptor 4 (TLR4) (which causes the sequestration and subsequent proteasome-independent degradation of PKC α) to block Fc ϵ RI-induced PLD-coupled, SK (sphingosine kinase)-mediated Ca²⁺ mobilization and NF κ B activation (Melendez *et al.* 2007) (Figure 9).

As previously described, signaling pathways elicited by KIT share several similar features with Fc ϵ RI-mediated events such as involvement of Src family PTKs, PLC- γ 1, PI3-K, calcium mobilization and MAPK-cascade activation (Figure 4). However, activated KIT neither phosphorylates LAT (Tkaczyk *et al.* 2004) nor recruits or activates Syk. The ability of KIT to potentiate Fc ϵ RI-dependent degranulation seems related to the involvement of NTAL and BTK and their capability to regulate PLC- γ 1-dependent calcium mobilization and PKC activation (Gilfillan & Beaven 2011). Of note, KIT can phosphorylate tyrosine residues in NTAL, which are different from those phosphorylated by Syk in Fc ϵ RI-activated cells (Iwaki *et al.* 2008). However, PLC- γ activation and calcium mobilization evoked by KIT are slower and of lower magnitude than those elicited by the Fc ϵ RI (Hundely *et al.*

2004). It is also clear that these signals by themselves cannot induce degranulation. Indeed, SCF by itself is unable to induce degranulation because of its inability to induce a detectable increase in tyrosine phosphorylation of LAT (Tkaczyk *et al.* 2004) and activation of PKC (Hundely *et al.* 2004). Finally, very recent data demonstrate that the G protein-coupled receptors GPCR kinase 2 (GRK2) modulates FcεRI signaling in MCs *via* at least two mechanisms. One involves GRK2-RH (regulator of G protein signaling homology domain, GRK2-RH) and modulates tyrosine phosphorylation of Syk (spleen tyrosine kinase), and the other is mediated *via* the phosphorylation of p38 and AKT (Subramanian *et al.* 2014).

1.5. Other mast cell receptors

Besides KIT and FcεRI, MCs express several other surface receptors that enable them to perform various functions including recognition of microbes, signaling to other immune cells, interactions with neurons and positive or negative modulation of other surface receptors (Pundir & Kulka 2010; Gilfillan & Beaven 2011; Sandig & Bulfone-Paus 2012; Suurmond *et al.* 2015).

Mast cells (MCs) express Toll-like receptors (TLRs) which are capable of binding microbial particles (Marshall 2004). Indeed, TLRs enable MCs to respond to products of gram-negative and gram-positive bacteria. Furthermore, the tissue origin of MCs and/or culture conditions can influence the expression of different TLRs (Marshall & Jawdat 2004). The most well-studied TLRs on MCs, TLR2 and TLR4, which have been reported to differentially activate human MCs (Varadaradjalou *et al.* 2003), and to act synergistically with antigen to enhance cytokine production (Gilfillan & Beaven 2011; Suurmond *et al.* 2015).

Mast cells (MCs) express also receptors for various cytokines, such as, among others IL-4, IL-9 and IL-10. They express also CD87 (urokinase receptor) and integrins receptors (Sperr *et al.* 1992; Sillaber *et al.* 1997; Agis *et al.* 1996; Valent *et al.* 2001; Ghannadan *et al.* 2002; Valent *et al.* 2010). The expression of activation-linked surface antigen CD88 (C5aR) on MCs depends on the localization (organ) or type of disease (Füreder *et al.* 1995; Agis *et al.* 1996; Valent *et al.* 2010). As an example, normal foreskin MCs express this antigen, whereas normal lung MCs lack it (Füreder *et al.* 1995; Agis

et al. 1996; Valent *et al.* 2001; Ghannadan *et al.* 2002; Valent *et al.* 2010). Besides, in patients with rheumatoid arthritis, synovial MCs harbor CD88, whereas CD88 is not expressed on MCs in osteoarthritis (Kiener *et al.* 1998; Valent *et al.* 2001).

Normal MCs do not express CD25 (IL-2 receptor (IL-2RA)), IL-3 receptor and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) (Valent *et al.* 1989; Agis *et al.* 1996; Valent *et al.* 2001; Scherthaner *et al.* 2005; Valent *et al.* 2010). In addition, MCs express a number of activation-related surface antigens such as CD63 and CD203c, and also chemokine receptors, or complement receptors (Bühning *et al.* 1999, Valent *et al.* 2001; Ghannadan *et al.* 2002; Scherthaner *et al.* 2005; Valent *et al.* 2010). However, the expression of some of these receptors depends on the differentiation stage. Thus, dependent on maturation, activation state, disease or localization, MCs are heterogeneous cells (Valent *et al.* 2001; Ghannadan *et al.* 2002; Valent *et al.* 2010), but they all invariably express the activation-linked surface antigen CD9, but also CD33 (Siglec-3), CD44 (the homing receptor Pgp-1), CD45 (leukocyte common antigen), the membrane cofactor protein CD46, the decay-accelerating factor CD55 and the membrane attack complex inhibitory factor CD59 (Valent *et al.* 1989; Valent *et al.* 1992; Valent *et al.* 2001; Ghannadan *et al.* 2002; Krauth *et al.* 2007; Valent *et al.* 2010; Teodosio *et al.* 2015).

Finally, apart from the inhibitory Fc γ RIIB, MCs express other activating high or low affinity receptors for IgG, Fc γ RI (CD64) and Fc γ RIII (CD16), which share the same γ subunit dimer with Fc ϵ RI, while their α -chains bind IgG. Their expression may vary according to the presence of cytokines, local environment, and species origin of the cells (Kraft & Kinet 2007. Draber *et al.* 2015).

1.6. Mast cell mediators

Activated MCs can release three classes of mediators: preformed (granule-bound) mediators, lipid-derived mediators, and cytokines and chemokines. The preformed mediators are stored in the secretory granules in the cytoplasm and are released within minutes after activation (Prussin & Metcalfe 2006; Metcalfe 2008; Amin 2012; da Silva *et al.* 2014). These mediators include histamine, the MC exclusively expressed serine proteases including tryptase, chymase, and carboxypeptidase,

Table II: MC mediators. TNF- α : tumor necrosis factor- α ; INF- α : Interferon- α ; MCP, monocyte chemotactic protein; MIF: macrophage inflammatory factor; GM-CSF: granulocyte monocyte-colony stimulating factor; b-FGF: fibroblast growth factor; NGF: nerve growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; SCF: stem cell factor; VEGF: vascular endothelial growth factor (Table adapted from Theoharides & Kalogeromitros 2006; da Silva *et al.* 2014).

Mediators	Main pathophysiologic effects
<u>Preformed</u>	
• Biogenic amines	
Histamine	Vasodilation, angiogenesis, mitogenesis, pain
5-Hydroxytryptamine (5-HT, serotonin)	Vasoconstriction, pain
• Chemokines	
IL-8, MCP-1, MCP-3, MCP-4, RANTES	Chemoattraction and tissue infiltration of leukocytes
• Enzymes	
Arylsulfatases	Lipid/proteoglycan hydrolysis
Carboxypeptidase A	Peptide processing
Chymase	Tissue damage, pain, angiotensin II synthesis
Kinogenases	Synthesis of vasodilatory kinins, pain
Phospholipases	Arachidonic acid generation
Tryptase	Tissue damage, activation of PAR, inflammation, pain
• Peptides	
Corticotropin-releasing hormone (CRH)	Inflammation, vasodilation
Endorphins	Analgesia
Endothelin	Sepsis
Kinins (bradykinin)	Inflammation, pain, vasodilation
Somatostatin (SRIF)	Anti-inflammatory action
Substance P (SP)	Inflammation, pain
Vasoactive intestinal peptide (VIP)	Vasodilation
Urocortin	Inflammation, vasodilation
Vascular endothelial growth factor (VEGF)	Neovascularization, vasodilation
• Proteoglycans	
Chondroitin sulfate	Cartilage synthesis, anti-inflammatory action
Heparin	Angiogenesis, nerve growth factor (NGF) stabilization
Hyaluronic acid	Connective tissue, nerve growth factor (NGF) stabilization
<u>Newly synthesized</u>	
• Cytokines	
Interleukins (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IL-16)	Inflammation, leukocyte migration, pain
INF-γ; MIF; TNF-α	Inflammation, leukocyte proliferation/activation
• Growth factors	
CSF, GM-CSF, b-FGF, NGF, VEGF	Growth of a variety of cells
• Phospholipid metabolites	
• Chemokines	
Leukotriene B4 (LTB4)	Leukocyte chemotaxis
Leukotriene C4 (LTC4)	Vasoconstriction, pain
Platelet-activating factor (PAF)	Platelet activation, vasodilation
Prostaglandin D2 (PGD2)	Bronchoconstriction, pain
• Nitric oxide (NO)	Vasodilation

proteoglycan and the lysosomal enzyme β -hexosaminidase which is ubiquitous to all MC subtypes in all species and quantification of the released β -hexosaminidase activity is often used as a measure of MC degranulation (Lundequist & Pejler 2011; da Silva *et al.* 2014). Currently, several studies suggest that numerous cytokines and growth factors are stored in MC granules along with other preformed mediators (Lundequist & Pejler 2011; da Silva *et al.* 2014). Table II presents preformed mediators of the MC as well as the mediators synthesized by this cell after its activation (Theoharides & Kalogeromitros 2006; da Silva *et al.* 2014).

Three main consequences of the activation of Fc ϵ RI are known and cause mediator release of the MCs, as indicated earlier. The first outcome of the cross-linking is the integration of the cytoplasmic granules with the plasma membrane causing within minutes the release of preformed mediators such as histamine, tryptase and chymase. The next step of the activation is the release of newly synthesized lipid mediators like leukotriene C₄ (LTC₄) or prostaglandin D₂ (PGD₂) caused through immediate synthesis of the membrane because of Ca²⁺ influx. Afterwards, a delayed release of the diverse cytokines, growth factors and chemokines via the IgE- or IgG-dependent stimulation or other activated gene expressions occurs (Metcalf *et al.* 1997; Prussin & Metcalfe 2006).

Especially, histamine has major effects on various cell types in physiologic and pathologic tissues (Jutel *et al.* 2009). Basophils (Bas) are the major source of histamine in the circulation, while MCs are the primary source of histamine in tissues (Graham *et al.* 1955; Valentine *et al.* 1955; Crivellato *et al.* 2011). Histamine and other released inflammatory mediators have an influence on vascular cells, smooth muscle cells, connective tissue cells, mucous glands and various inflammatory cells. Histamine plays a special role after local infection, where it causes an increased recruitment of diverse inflammatory cells (Amin 2012). Through its binding on histamine receptors (HRs; H₁R and H₂R), histamine has effects on endothelial cells, smooth muscles (contraction), nerve endings and mucous secretion (Olafsson 1985). Histamine is metabolized into to N-methyl histamine, methylimidazole acetic acid, and imidazole acetic acid, all metabolites being excreted by the kidneys.

Histamine in secretory granules is found in anionic association with acidic residues of the glycosaminoglycan side chains of heparin and chondroitin sulphate E and dissociates in extracellular

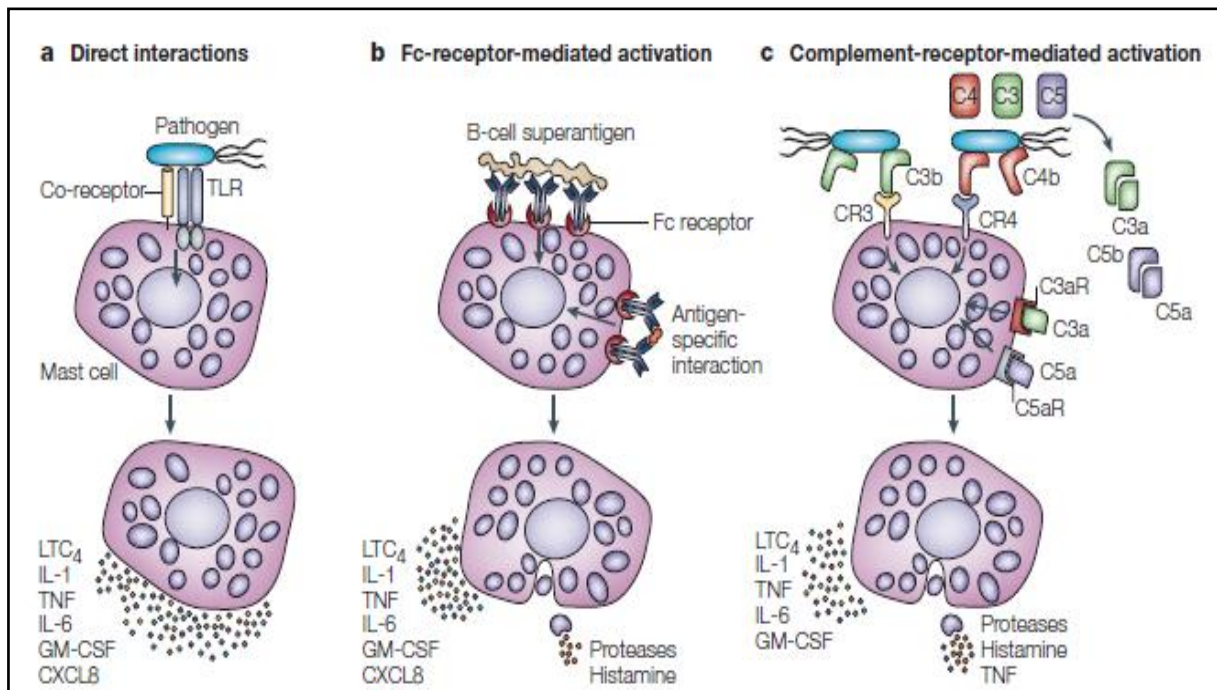


Figure 10: The variety of MC activation pathways by pathogens ensures the effective role of sentinel of MCs. CXCL8, CXC-chemokine ligand 8; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LTC₄, leukotriene C₄ (Figure adapted from Marshall 2004).

fluids by exchanging with sodium ions. The release of histamine stored in the cytoplasmic granules of MCs and Bas is caused by a vesicular transport through cell degranulation induced by various stimuli such as chemical substances, endogenous mediators as well as through immune mechanisms (Dvorak *et al.* 1996; Jutel *et al.* 2009; Crivellato *et al.* 2010; Amin 2012).

Through the binding of the receptor FcεRI, MCs undergo a conformational change. First, intracellular degranulation chambers are shaped through granule membrane fusions as well as the solubilisation of the granules before they are secreted. Second, formed granule vesicles travel from the storage granule compartment to the plasma membrane through the cytoplasm, where the granule vesicles fuse with the membrane, leading to the release of the preformed mediators (Dvorak *et al.* 1996; Crivellato *et al.* 2010; Amin 2012).

1.7. Mast cell activators

MCs undergo activation by direct and indirect mechanisms upon the exposure to pathogens (Figure 10). Direct mechanisms include TLR-mediated interactions between MCs and pathogen which frequently do not result in MCs degranulation but lead to cytokine, chemokine and lipid mediator production (Figure 10a). Indirect activation of MCs includes Fc receptor-mediated activation (Figure 10b), and complement receptor-mediated activation (Figure 10c). Fc receptor-mediated activation (activation *via* crosslinking of the FcεRI) leads to MC degranulation and production of newly generated mediators. Complement receptor-mediated activation of MCs through the receptor for C5a leads to MC degranulation. Complement receptor mediated activation can occur through various receptors for complement components (such as CR3, the receptor for complement component 3b, C3b), and receptors for C3a (C3aR) (Marshall 2004).

In addition, it is well known that the pattern and the extent of mediators released upon MC degranulation and activation depend on the subtype and localization of the MCs (cytokine milieu) and the type and intensity of the stimulus and triggers (Gilfillan & Tkaczyk 2006). For example, the FcεRI-induced mediator secretion can be augmented by activation of MCs *via* the specific SCF receptor and additional activation of Bas through the IL-3 receptor (Sperr *et al.* 1993; Okayama *et al.*

Table III: The major MC receptors involved in host defense (Table adapted from Marshall 2004).

Receptor class	Examples of receptors	Examples of ligands
Direct receptors for pathogen products		
• Toll-like receptors (TLRs)	TLR1	Lipopeptide
	TLR2	PGN, zymosan and some LPS
	TLR3	Double -stranded RNA
	TLR4	LPS and F protein of RSV
	TLR6	PGN and zymosan
	TLR9	Bacterial DNA and CpG-containing DNA
• Manosylated receptors	CD48	FimH
Indirect receptors for products of immune responses to pathogens		
• Fc receptors	FcεRI	IgE
	FcγRI, FcγRII and FcγRIII	IgG
• Complement receptors	CR2, CR4 and CR5	Complement components
	C5aR and C3aR	Complement spilt products
• Protease-activated receptors	PAR2	Mast cell proteases
• Cytokine receptors	IL-1 and INF-γR	Immunoregulatory cytokines
	IL-10R and IL-12R	Immunoregulatory cytokines
• Chemokine receptors	CCR3, CCR5 and CXCR4	Mast cell chemoattractants

1993; Valent 1994; Gebhardt *et al.* 2002; Valent *et al.* 2002). In addition to aggregation of FcεRI, MCs are also activated by C3a and C5a *via* the C3a receptor and the C5a receptor (CD88) (Füreder *et al.* 1995; Valent *et al.* 2001; Prussin & Metcalfe 2006), and by IgG *via* FcγRI (Tkaczyk *et al.* 2002). MCs might also be activated *via* TLRs. For instance, TLR3-mediated activation of human MCs induces them to produce type 1 interferons (Kulka *et al.* 2004).

Mast cell triggers include IgG, IgE+antigen, corticotropin-releasing hormone (CRH), anaphylatoxins, IL-1, immunoglobulin-free light chains, lipopolysaccharide (LPS), NGF, neurotensin (NT), SCF, substance P (SP), superantigens, urocortin (Ucn), vasoactive intestinal peptide (VIP), viral DNA sequences (Amin 2012). Additionally, MC degranulation occurs after physical stimuli such as heat and laser (Zhang *et al.* 2012; Hügler 2014).

1.8. Mast cell functions in physiology and in pathological situations

1.8.1. Mast cell function in host defense, innate and acquired immunity

Mast cells play a crucial role in host defense by modulating innate and adaptive immune responses because of their intrinsic capacity to release a wide range of proinflammatory mediators (Abraham & Malaviya 1997; Abraham & Arock 1998; Galli *et al.* 1999; Wedemeyer *et al.* 2000; Galli 2000; Henz *et al.* 2001; Galli *et al.* 2005). In addition, these cells express various receptors including CD43 (leukosialin), CD80, CD86, and intercellular adhesion molecule receptors (ICAM-1 and ICAM-3) enabling them to interact with B and T lymphocytes. Table III shows the major receptors of MC involved in host defense.

Since MCs are present at strategic locations enabling them to encounter pathogens, they have been well established to play a role in the defence against bacteria, certain parasites (mainly intestinal helminth parasites), and possibly against fungal and viral infections (Malaviya *et al.* 1996; Supajatura *et al.* 2001; Féger *et al.* 2002; Marshall 2004; Sutherland *et al.* 2008; Shelburne *et al.* 2009). Recognition of pathogens *via* Toll-like receptors (TLR) has been shown to contribute to MC responses to pathogens (Supajatura *et al.* 2001).

Binding of microbial compounds to TLRs on MCs leads to release of TNF, IL-1, IL-6, and IL-8 and enhances the migration of effector cells to the infected tissues (Marshall 2004). Moreover, murine and human MCs have been reported to produce IFNs type I after exposure to double-stranded RNA and/or virus, the former *via* specific interactions with TLR3. These data suggest that MCs contribute to innate immune responses to viral infection *via* the production of IFNs type I, which involves NF- κ B, p38, and C-Jun NH2-terminal kinase and MAP kinase (Kulka *et al.* 2004).

It has been shown that TNF- α released by MCs can control bacterial infection in a murine model of acute septic peritonitis (Echtenacher *et al.* 1996), while nematode infections in mice lead to accumulation of mucosal MCs in the gut with release of MC protease 1 (the protease that is unique to mucosal MCs of mice) (Knight *et al.* 2000). Beside the effect of MC released TNF- α , CB-derived human MCs can kill and phagocyte various gram-negative and gram-positive bacteria (Arock *et al.* 1998).

Thrasher *et al.* have recently documented the role of mucosal rat MCs in parasite expulsion during the infection with *Trichinella spiralis*. Rat BM-derived MCs and rat basophilic leukemia cell line (RBL-2H3) were used in that study as models for mucosal MCs and found to be activated without degranulation in response to parasite glycoproteins, demonstrating these models in the contexts of innate and adaptive responses to *T. spiralis* (Thrasher *et al.* 2013).

Furthermore, parasite infections are often associated with increased levels of circulating Bas and eosinophils, increased serum levels of IgE (which enhanced expression of Fc ϵ RI on Bas and MCs) and increased numbers of MCs and/or Bas in the affected tissues (Metcalf *et al.* 1997; Galli *et al.* 1999; Galli 2000; Wedemeyer *et al.* 2000; Williams & Galli 2000).

Regarding the role of MCs in acute viral infections, to date, little is known (Abraham & St John 2010; St John & Abraham 2013). However, evidence has recently emerged that MCs significantly influence immunity and pathogenesis during dengue virus (DENV) infection (St John 2013). MCs degranulate within minutes after exposure to DENV, followed by *de novo* cytokine production in the subsequent hours. Detection of chymase in the serum of DENV patients with acute infection also demonstrates that MC degranulation occurs *in vivo* during the course of clinically significant infections (St John *et*

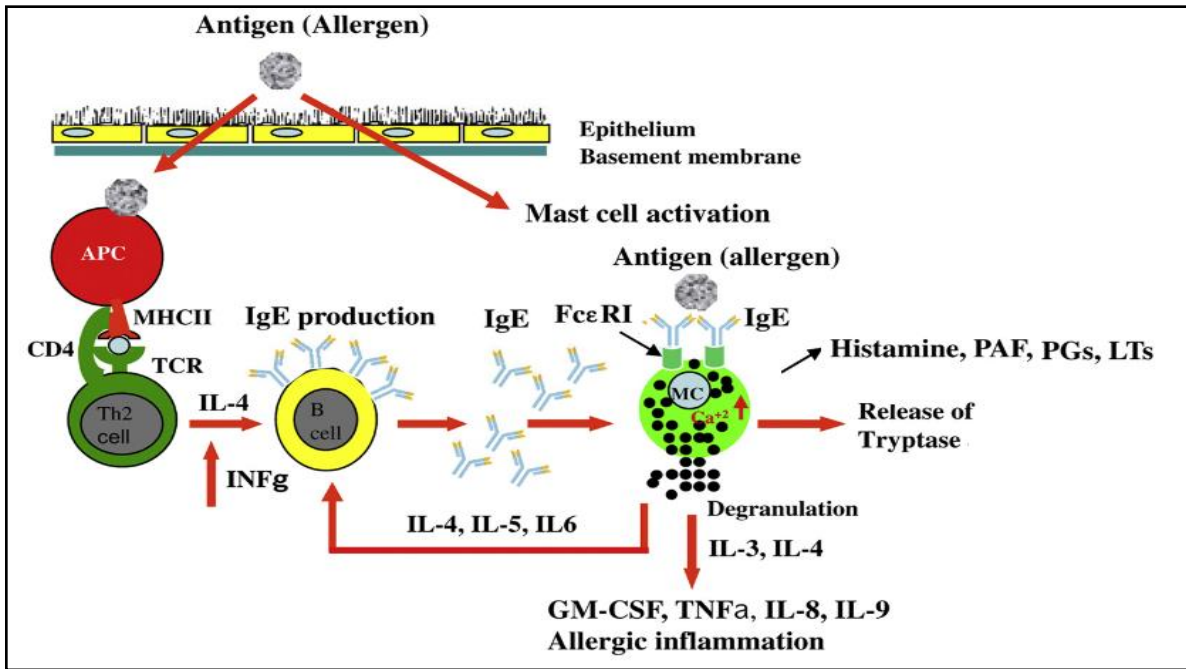


Figure 11: The role of MCs in type 1 hypersensitivity. APC, antigen presenting cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LTs, leukotrienes; MHCII, major histocompatibility complex class II; PAF, platelet activating factor; PGs, prostaglandins; TCR, T cell receptor, TNF, tumour-necrosis factor (Figure adapted from Amin 2012).

al. 2011). The degranulation is thought to have a protective role and to help viral clearance (St John 2013). However, MC-induced vascular leakage at a systemic level can contribute to DENV pathogenesis, both during primary and secondary infection (St John 2013).

1.8.2. Mast cell function in allergy, asthma, tissue remodeling and fibrosis

Immediate hypersensitivity occurs within minutes after exposure to allergen, whereas late phase occurs within hours, and in chronic phase symptoms relapse and remit overtime as in asthma (Galli *et al.* 1999). In the context of allergy, it is well known that MCs are involved in immediate hypersensitivity, late-phase allergic inflammation and chronic inflammation associated with diseases such as asthma.

As mentioned above, during an allergic event, B cell-released IgE binds to the FcεRI on MC surface via their Fc segment. This lets their Fab, antigen-binding segment, free to bind antigens (Klein *et al.* 1989; Metcalfe *et al.* 1997; Church & Levi-Schaffer 1997; Nakanishi 2010; Amin 2012). A subsequent exposure to the same allergen crosslinks the MC-bound IgE and thus triggers the release of preformed cytokines, histamine and prostaglandins (Figure 10) (Amin 2005; Nakanishi 2010; Amin 2012).

Since MCs are positioned at the interface of the external environment in the skin and at mucosal surface, they are the central effector cells in the early allergic inflammatory responses. These cells encounter antigens that elicit allergic events (Galli & Wershil 1996). Figure 11 shows the interaction between FcεRI associated with IgE and the multivalent allergen, leading to the immediate release of contents of MC secretory granules, including preformed mediators mentioned above such as histamine, tryptase, chymase and proteoglycan which directly influence the surrounding tissue. Chymase, cathepsin G and carboxypeptidase activate a rebuilding of the connective tissue matrix by metalloproteases. Histamine and heparin increase vascular permeability and trigger smooth muscle contractions. Newly synthesized mediators including prostaglandin D2 (PGD2) (the products of endogenous arachidonic acid metabolism) and leukotrienes such as LTB4, LTC4 and LTE4 are also released and are responsible for mucus hypersecretion and maintenance of inflammatory reactions. Of

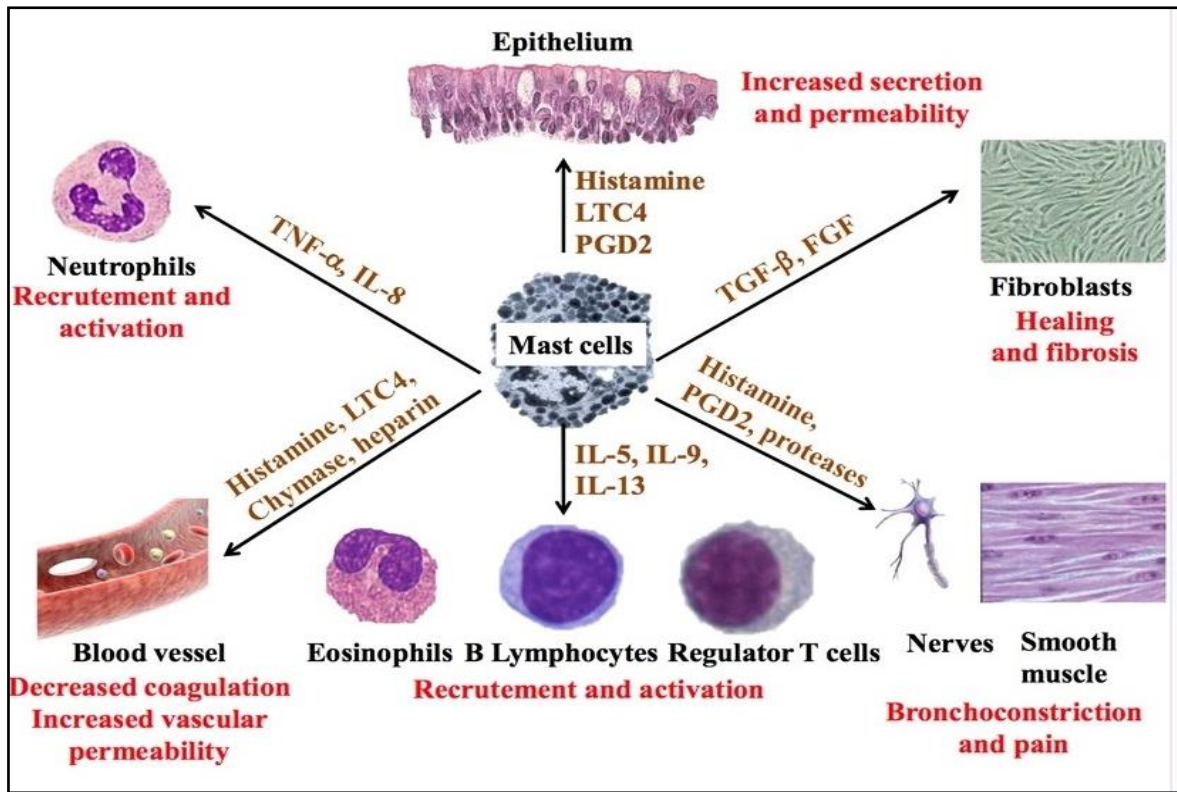


Figure 12: A schematic representation of the biological effects of the major MC mediators on surrounding cells and tissues. Target cells and tissues are in black, MC-derived mediators are in brown, and biological effects are in red (Figure from Arock *et al.* 2015).

Table IV: Inflammatory diseases involving MCs (Table adapted from Arock *et al.* 2015).

Disease	Pathophysiologic effects
Asthma	Bronchoconstriction, pulmonary inflammation
Atopic dermatitis	Skin vasodilation, T-cell recruitment, inflammation, itching
Coronary artery disease (CAD)	Coronary inflammation, myocardial ischemia
Chronic rhinitis	Nasal inflammation
Chronic prostatitis	Prostate inflammation
Fibromyalgia	Muscle inflammation, pain
Interstitial cystitis	Bladder mucosal damage, inflammation, pain
Migraine	Meningeal vasodilation, inflammation, pain
Multiple sclerosis	Increased blood–brain barrier permeability, brain inflammation, demyelination
Neurofibromatosis	Skin nerve growth, fibrosis
Osteoarthritis	Articular erosion, inflammation, pain
Rheumatoid arthritis	Joint inflammation, cartilage erosion

particular interest in humans is the production of TNF α , IL-4, IL-5, IL-6, IL-1 β and IL-13 (Abraham 1997; Arock *et al.* 1998; Holgate 2007; Nakanishi 2010; Amin 2012).

The late onset reaction again is initiated by preformed and newly synthesized MC mediators. By secreting cytokines like TNF α , LTB₄, IL-4 or histamine, endothelial cells are activated and upregulate expression of adhesion molecules such as P-selectin, E-selectin, vascular cell adhesion molecule (VCAM1) and ICAM-1 on their surface. Leucocytes such as neutrophils, monocytes and CD4⁺ T cells can bind to these molecules and migrate into the tissues by transmigration (Koh *et al.* 1993; Robinson *et al.* 1993; Montefort *et al.* 1994; Amin 2012). MCs are also involved in late phase hypersensitive responses. In MC deficient mouse model, late and immediate phases of IgE-mediated cutaneous inflammation appear dependent on MCs (Galli *et al.* 1992).

In addition, MCs seem to play a crucial role in airway remodeling by releasing tryptase onto smooth muscle and epithelium, and may play a role in skin tissue remodeling by releasing chymase in an IgE-dependent manner in allergic diseases (Amin 2005) (Figure 11). Furthermore, Amin showed that MCs are specifically localized within or close to airway smooth muscle bundles in patients with allergic asthma whereas little or no MCs are found in the airways of healthy controls (Amin 2012). These cells also have an effect on epithelial damage, and on basement membrane thickening in patients with allergic asthma (Amin *et al.* 2000; Oh 2005; Amin 2012), allergic rhinitis (Amin *et al.* 2001) and middle ear infection in allergy patients (Hurst 1999). Figure 12 depicts the biological effects (labeled in red) of major MC-derived mediators including histamine, heparin, proteases, chymase, interleukins (IL-4, IL-5, IL-8, IL-9, IL-13), LTC₄, FGF, PGD₂, TGF- β , and TNF- α (labeled in brown) on target cells and tissues (labeled in black) (Arock *et al.* 2015).

Besides, increasing evidence indicate that MCs are critical for the development of inflammatory diseases, especially in the pathogenesis of arthritis, asthma, chronic dermatitis, and coronary artery disease (CAD). Table IV represents the inflammatory diseases which involve MCs (Theoharides & Cochrane 2004; Theoharides & Kalogeromitros 2006). However, unlike allergic reactions, MCs rarely degranulate during autoimmune (Benoist & Mathis 2002) or inflammatory (Woolley 2003) processes. The only explanation for MC involvement in non allergic processes may be through “differential” or

“selective” secretion of mediators without degranulation (Theoharides *et al.* 1982; Theoharides *et al.* 2007).

Finally, Suurmond *et al.* have more recently documented that the combination of IgE- and TLR-mediated activation greatly enhances cytokine production by MCs, but not their degranulation (Suurmond *et al.* 2015). Whereas MC degranulation is important for acute-phase allergic responses, cytokine production is considered to be important for the more inflammation related, late-phase responses (Naclerio 1997; Larché *et al.* 2006) Therefore, the presence of TLR ligands may specifically enhance the late-phase responses in allergy (Suurmond *et al.* 2015).

Of note, as described above, MCs are involved in the parasitic immune response and intrahepatic schistosomiasis is a well-known cause of liver fibrosis (Andrade 2009). MCs are necessary for the development of eosinophils, IL-5 expression and liver fibrosis in schistosomiasis-infected animals (Sabin *et al.* 1996). MCs have been implicated in the pathogenesis of fibrosis in various organs. For example, MCs drive cardiac fibrosis after ischaemic cardiopathy or pressure overloaded heart model (Kong *et al.* 2013). Conversely, MC deficient mice are protected from cardiac fibrosis during heart failure (Hara *et al.* 2002).

Besides, in renal fibrosis due to IgA nephropathy, increased numbers of MCs are found in the kidney and correlate with fibrosis, whereas under normal circumstances MCs are not found in the kidney (Roberts & Brenchley 2000). The role of MCs, and SCF in tubulointerstitial lesions in IgA nephropathy has been recently reported (Liu *et al.* 2010; Li *et al.* 2010). MCs are also increased in the lung tissue of patients with idiopathic pulmonary fibrosis (Tuder 1996).

Interestingly, MCs contain profibrotic cytokines such as transforming growth factor-beta (TGF- β), platelet derived growth factor (PDGF) and GM-CSF in their vesicles. MC Chymase cleaves the latent form of transforming growth factor-beta (TGF- β) from cell membrane (Taipale *et al.* 1995). TGF- β is considered as a main driver for collagen production of fibroblasts and their differentiation into myofibroblasts. Expression of MC chymase in human patients with idiopathic pulmonary fibrosis is increased (Hirata *et al.* 2007), the secretion of histamine or the activation of the renin-angiotensin system stimulates the production of collagen by fibroblasts (Veerappan *et al.* 2013).

In addition, it has been shown that the number of skin MCs is increased in patients with systemic sclerosis (SSc), which is considered as the prototype of all fibrotic diseases (Hawkin *et al.* 1985). However tryptase serum is not elevated in these patients or in other forms of fibrosis. MCs are the main source of TGF- β in the skin of SSc patients. Hügle's group showed the transgranulation cell-cell contact as a mechanism of fibrosis in SSc patients. In this case, TGF- β present in the MC vesicles is being transferred from MCs to fibroblasts (Hügle *et al.* 2012).

Finally, IL-4 and GM-CSF stored in MCs participate in the differentiation of PB derived monocytes into functionally active and contractible myofibroblasts (Binai *et al.* 2012).

1.8.3. Mast cell function in vascular diseases

MCs are involved in vascular injury and atherosclerosis (Kelly *et al.* 2000). The content of MC granules released upon activation have anticoagulant as well as thrombogenic functions (Szczeklik *et al.* 1988; Szczeklik 2000). Their number increased in the shoulders of atherosclerotic plaques where MCs are associated with plaque rupture culminating in luminal thrombosis (Jeziorska *et al.* 1997). Increased MC number was also reported by Kovanen *et al.* in the atheromatous plaque rupture in patients died from acute myocardial infarction (Kovanen *et al.* 1995). In addition, MCs have been reported to produce tissue plasminogen activator (Sillaber *et al.* 1999; Oh 2005) and plasminogen activator inhibitor-1 (Cho *et al.* 2000).

More recently, Bot *et al.* have reported that MCs are effectors in atherosclerosis through the release of various mediators, such as the MC specific proteases chymase and tryptase, growth factors, histamine, and chemokines, which have detrimental effects on immediate surroundings in the vessel wall. This leads to matrix degradation, apoptosis, and enhanced recruitment of inflammatory cells, thereby actively contributing to cardiovascular diseases (Bot *et al.* 2014).

1.8.4. Mast cell involvement in autoimmunity and other diseases

Several investigations have underlined the key role of MCs in coordinating the early phases of autoimmune diseases, especially those involving autoantibodies. This role of MCs was prompted by

studies on multiple sclerosis which is an autoimmune demyelinating disease of central nervous system (CNS) mediated by T helper cells (Th1) and/or (Th17), which cross the blood-brain barrier, and on its animal model, experimental allergic encephalomyelitis (EAE) (Steinman 2001). Of note, mice lacking MCs were found to develop EAE later and less severely than do the control mice in response to injection of myelin oligodendrocyte glycoprotein. Reconstitution of the MC population in MC lacking mice restored induction of early and severe disease to wild-type levels (Secor *et al.* 2000; Costanza *et al.* 2012).

Furthermore, various studies have reported a correlation between the number/distribution of MCs in the central nervous system (CNS) and the development of multiple sclerosis and EAE (Olsson 1974; Secor *et al.* 2000; Brown 2002; Theoharides *et al.* 2008). In addition, increased tryptase level was observed in the cerebrospinal fluid of patient with multiple sclerosis (Rozniecki 1995) and the existence of MC related transcripts encoding histamine, proteases, FcεRI and other inflammatory mediators was reported in the same biological fluid (Chabas *et al.* 2001; Benoist & Mathis 2002; Lock *et al.* 2002).

Also, always in multiple sclerosis, brain MCs, which are perivascularly located, are activated by neural factors such as substance P and myelin basic protein and can secrete numerous proinflammatory and vasoactive molecules that may increase the blood-brain barrier permeability, thus facilitating the entry of inflammatory cells to the CNS (Theoharides *et al.* 2008; Frenzel & Hermine 2012).

Moreover, brain MCs can stimulate activated T cells coming in contact with them, including Th17 at the blood-brain barrier. Astrocytes are also activated after the interaction with MCs *via* CD40/CD40 ligand complex. The activated astrocytes contribute to MC degranulation through releasing IL-33 and IL-1β (Theoharides *et al.* 2008; Carlson *et al.* 2008; Frenzel & Hermine 2012).

Besides, MCs are effector cells in rheumatoid arthritis (Benoist & Mathis 2002; Hueber *et al.* 2010; Merluzzi *et al.* 2014; Hügler 2014). Activating IgG receptors on MCs are engaged by IgG autoantibodies (Merluzzi *et al.* 2014), which may explain the activation of MCs in this autoimmune disease. The proinflammatory cytokine IL-17A stored in MC vesicles as well as preformed MC TNF-α

Table V: Correlation between MC infiltration and tumor outcome (Table adapted from Rigoni *et al.* 2014).

Tumor	Prognosis	Brief description of MC activity
Breast cancer	Good	Stromal MC infiltration, even in small quantity, is a good prognostic marker in breast cancer.
	Good	MCs are recruited by stem cell factor and produce an immunosuppressed tumor microenvironment.
Colorectal cancer	Poor	High micro vessels density and MC number is associated with metastases and shorter survival.
	Poor	High density of MCs at the invasive margin of tumors favors colorectal cancer progression.
	Poor	In polyps, MCs and their precursors sustain inflammation and progression to cancer.
Esophagus squamous cell carcinoma	Good	MCs sustain antitumor immunity producing IL-17 and predict a favorable prognosis.
	Poor	High MCs number at the invasive edge of tumor favors tumor progression and decreases survival rate post-surgery.
Gastrointestinal cancer	Poor	Number of MCs expressing tryptase is correlated with lymph node metastatization post-surgery.
Hepatocellular carcinoma	Good	High density of MCs predicts late recurrence.
Lung cancer	Good	MCs infiltration of non-small cell lung cancer is associated with increase in 5-year survival, independently of tumor stage.
	Poor	In lung adenocarcinoma, MC density correlates with angiogenesis and poor prognosis.
Malignant melanoma	Poor	Higher MC number found in invasive melanoma compared with benign nevi or in situ melanoma.
	Poor	High MC number correlates with high microvessels density and poor prognosis.
Neurofibromatosis	Poor	MCs sustain neurofibromas initiation and progression.
Pancreatic cancer	Poor	Higher MCs number in pancreatic cancer than in normal pancreas; MCs number correlates with metastatic potential.
	Poor	Tumor stroma infiltration by MCs is associated with poor prognosis.
	Poor	High MCs number at the tumor edge correlates with invasion, metastasis and tumor staging.
Prostate cancer	Good	Intratumoral MCs negatively regulate angiogenesis and tumor growth.
	Poor	Peritumoral MCs stimulate the expansion of tumor.
	Poor	MCs favor the progression to adenocarcinoma but are absent in neuroendocrine tumor foci.
Primary cutaneous Lymphoma	Poor	High MCs number at tumor periphery is associated with progressive disease
Diffuse large B-cell lymphoma	Good	High MCs count is associated with a favorable disease outcome.
Hodgkin's lymphoma	Poor	High number of infiltrating MCs is associated with worse relapse-free survival.
Splenic marginal zone Lymphoma	Poor	MCs interaction with stromal cells impact on disease progression.
Angioimmunoblastic T-cell lymphoma	Poor	Accumulation of MCs helps neoangiogenesis and characterizes angio-immunoblastic T-cell lymphomas.

play a crucial role in rheumatoid arthritis (Hueber *et al.* 2010; Hügler 2014).

MCs are also implicated in Waldenström macroglobulinemia (WM), a distinct B cell lymphoproliferative disorder which is characterized by BM infiltration with lymphoplasmacytic cells (LPCs), along with the presence of an IgM monoclonal gammopathy (Ho *et al.* 2008). Bone marrow MCs provide important growth and survival signals to WM LPCs through multiple TNF-family ligands, including CD40L (CD154), a proliferation-inducing ligand (APRIL), and a B-lymphocyte stimulator factor (BLYS) (Tournilhac *et al.* 2006).

Additionally, The role of MCs and MC-derived mediators has been evaluated in autoimmune glomerulonephritis (Monteiro *et al.* 2015). mMCP-4 chymase, contrary to the global anti-inflammatory action of MCs, has been reported to aggravate the glomerulonephritis by promoting kidney inflammation (Scanduzzi *et al.* 2010).

1.8.5. Mast cell involvement in cancer

MCs are also linked to tumor invasion (Gounaris *et al.* 2007). Indeed, MC infiltration has been reported in several types of human tumors and in animal cancer models. Furthermore, the wide diversity in type, grade and stage of human tumors, as well as the poorly characterized distribution of MCs in tumors, present a paradigmatic situation and do not allow the unambiguous classification of MC function as pro- or anti-tumorigenic elements. Table V represents the correlation between MC infiltration and tumor outcome.

However, it is well known that MCs are recruited into the tumor microenvironment by chemotactic factors released by cancer cells. MC mediators may thus influence cancer cell survival and also have a role in the lymphangiogenesis, angiogenesis, invasion and metastasis through extracellular matrix (ECM) remodeling (Maltby *et al.* 2009; Marone *et al.* 2015). In addition, MCs interact with other tumor-infiltrating immune cells. For instance, MCs mobilize and modulate the activity of T cells, regulatory T (Treg) cells, antigen-presenting cells (APCs) and myeloid-derived suppressor cells (MDSCs). Particularly, MCs enhance T cell migration to the tumor microenvironment and change the phenotype of Treg cells. However, Treg cells also inhibit MC degranulation and allergic responses

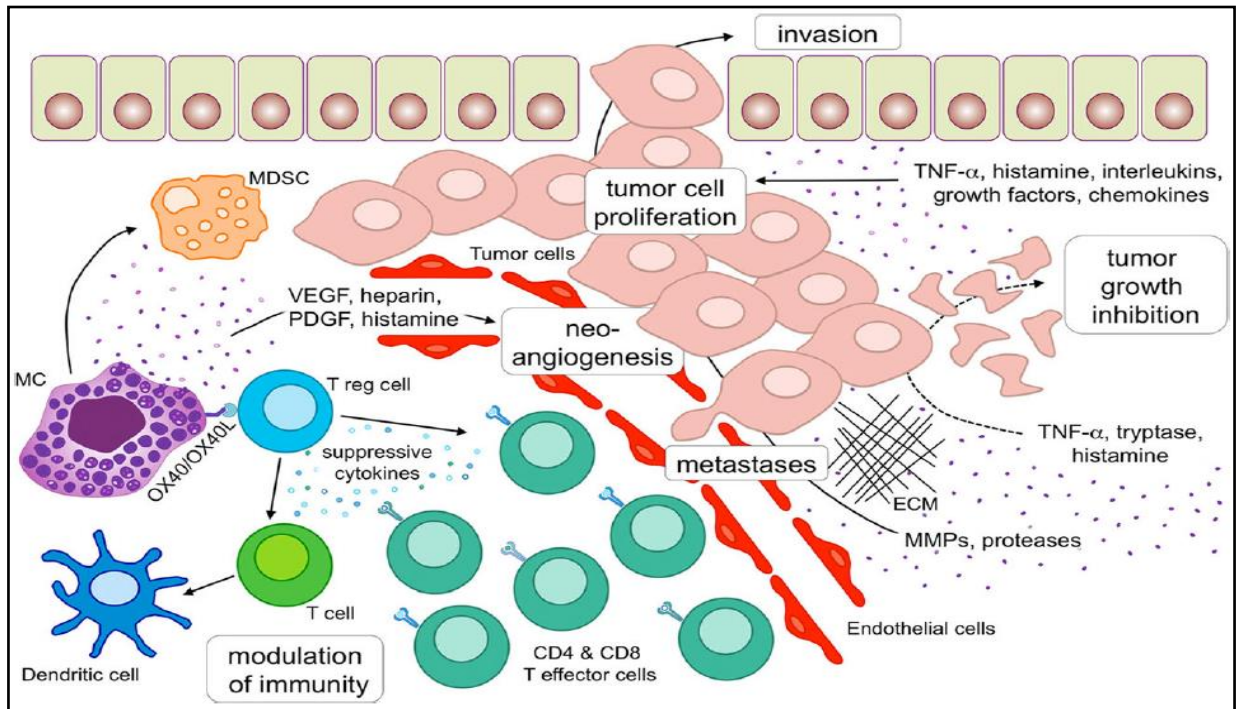


Figure 13: The complex network of interaction between MCs and other components of the tumor microenvironment (Figure adapted from Rigoni *et al.* 2014).

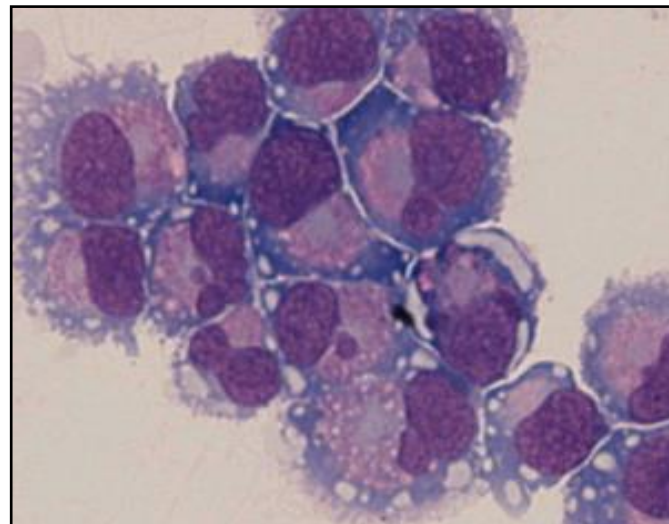


Figure 14: Morphological aspect of HMC-1 cells after May-Grünwald Giemsa (MGG) staining. Magnification x200.

through cell-cell contact (OX40/OX40ligand crosstalk between MCs and Treg cells) or by the production of soluble factors (Gri *et al.* 2008; Piconese *et al.* 2009; Gri *et al.* 2012).

In addition, MCs were recently found to boost MDSC activity and contribute to the development of tumor-favoring microenvironment (Danelli *et al.* 2015). Figure 13 summarizes the complex crosstalk between MCs and other immune cells in tumor microenvironment.

1.9. Human mast cell lines

Isolation of tissue MCs is a costly and difficult procedure, with a poor yield. To circumvent such drawbacks, MC lines are therefore used. However, there are only limited numbers of human MC lines facilitating the *in vitro* study of human MC biology. Indeed, before the isolation of the ROSA cell lines in our laboratory, only 4 human MC lines were described, namely HMC-1, LAD, LUVA, and MCPV-1 cell lines.

1.9.1. The human mast cell line HMC-1 and its two subclones (HMC-1.1 and HMC-1.2)

The human MC line (HMC-1) was derived from the PB of a patient with a MCL (Butterfield *et al.* 1988). HMC-1 cells are immature cells which stain metachromatically by toluidine blue, lack T and B lymphocyte as well as myeloid cell markers, and do not express IgE receptors. Figure 14 shows May-Grünwald Giemsa staining (MGG) of HMC-1 cells. In addition, they contain low levels of histamine, and chloroacetate esterase, aminocaproate esterase and tryptase activities (Butterfield *et al.* 1988).

In HMC-1 cells, KIT receptor was found to be constitutively phosphorylated on tyrosine residues, activated, and associated with PI3-K in the absence of SCF. Sequencing of whole coding region of *KIT* cDNA revealed that *KIT* genes of HMC-1 cells are composed of a normal WT allele and a mutant allele with two activating point mutations resulting in intracellular amino acid substitutions of G560 for V and V816 for D (Furitsu 1993; Kanakura *et al.* 1994). These mutations are responsible of SCF-independent constitutive phosphorylation of KIT (Furitsu 1993). Although the HMC-1 cell line had been widely employed for *in vitro* studies of human MC biology (Butterfield *et al.* 1988; Akin *et al.* 2003), being growth factor independent, the cells lack surface FcεRI expression (Nilsson *et al.* 1994;

Guhl *et al.* 2010), which limits their utility, especially in examining allergic mechanisms.

Since HMC-1 cells show immature characteristics of MCs and their relevance to normal MC function is questionable, Weber *et al.* have described and generated a subclone (5C6) of HMC-1 cells, by limiting dilution method (Weber *et al.* 1996). This clone has been published to represent a more mature MC than the original HMC-1 cell line, expressing MC markers more strongly than the original cells, as well as FcεRI. However, this subclone shows several limitations, and at least in our hands, is unable to degranulate after cross-linking of FcεRI.

More recently, two subclones of HMC-1, namely HMC-1(560) or HMC-1.1 and HMC-1(560,816) or HMC-1.2, were documented with different phenotypes and designated by the locations of specific mutations in the *KIT* protooncogene. Both sublines have a heterozygous T to G mutation at codon 560 in the JMD of the *KIT* gene causing an amino acid substitution of G560 for V. In contrast, only HMC-1.2 cells have the *KIT* D816V mutation causing a substitution of V for D in the intracellular KD (Sundström *et al.* 2003). HMC-1.1 cells are heterogeneous in size and exhibited some homotypic aggregation. They are also more adherent than HMC-1.2 cells, which grew in single cell suspension. HMC-1.2 cells are smaller in size and tend to be more homogeneous. Finally, the presence of *KIT* D816V mutation seems to confer an advantage to the cells in terms of proliferation rate (Sundström *et al.* 2003).

HMC-1.1 cells are sensitive to imatinib, while HMC-1.2 cells are resistant to imatinib but respond to dasatinib (Akin *et al.* 2003; Zermati *et al.* 2003; Schittenhelm *et al.* 2006). Besides, inhibition of kinase activity in *KIT* KD mutations was found to restore cell surface expression of the mutant receptor. As an instance, the *KIT* D816V in HMC-1.2 was found to relocalize to cell surface after dasatinib treatment (Bougherara *et al.* 2013).

LYN and BTK are phosphorylated and activated in both HMC-1.1 and HMC-1.2 cell lines in a *KIT*-independent manner as well as in neoplastic MCs in advanced SM. SiRNAs against LYN and BTK block the survival of HMC-1.1 and HMC-1.2 cell lines. Dasatinib was found to block also LYN and BTK activation in neoplastic MCs while bosutinib inhibits LYN and BTK activation without decreasing *KIT* kinase activity (Gleixner *et al.* 2011).

Moreover, the activation of PKC was found to have different effects in HMC-1.1 and HMC-1.2 cell lines as it translocates to nucleus only in HMC-1.2 cells upon stimulation with phorbol myristate acetate (PMA) and this may be related to the presence of the KIT D816V activating mutation in this cell line (Tobío *et al.* 2011).

STAT3 and STAT5 have been shown to be critical for cell proliferation elicited by KIT D816V in cell lines as well as in MCs from patients with systemic mastocytosis (SM) (Ning *et al.* 2001; Harir *et al.* 2008; Baumgartner *et al.* 2009). Chaix *et al.* have recently clarified the pathway linking KIT receptor to STAT activation and reported that STAT1, STAT3 and STAT5 are activated downstream molecules of KIT D816V mutant. Indeed, KIT D816V mutant can directly phosphorylate STAT1, 3 and 5 *in vitro* at tyrosine residues Y701, Y704, and Y694 respectively. Interestingly, STAT5 is transcriptionally active in the HMC-1 cell line, whereas the transcriptional activity of both STAT1 and STAT3 is absent. In addition, JAK3, which is activated downstream of KIT, has been involved in the phosphorylation of STAT proteins in HMC-1 cells (Chaix *et al.* 2011).

The lack of FcεRI in HMC-1 cells has led many researchers to use less physiological stimuli, such as calcium ionophores and phorbol esters to examine MC activation using these cell lines. Results of such studies are undoubtedly useful but provide limited insights into MC activation in allergic inflammation. For that, Xia *et al.* have recently generated and characterized a HMC-1 cell line which has been stably transfected with the human FcεRIα subunit, HMCα (Xia *et al.* 2011). HMCα cells express FcεRI that is functional and capable of binding human IgE. Incubation of these cells with human IgE over a period of 2-3 days leads to a stabilization of FcεRI on the cell surface (Xia *et al.* 2011).

1.9.2. LAD cell lines

LAD cell lines were developed in 2003 from a 44-year-old male with MC sarcoma/leukemia. Indeed, the BM-derived MNCs from this patient were cultured in serum free media (SFM) with SCF, with or without IL-6 and IL-3 (during the first week), resulting in generation of five cell lines (LAD1-5) that were maintained in SFM and SCF. LAD cells (both LAD-1 and LAD-2) present with many well-

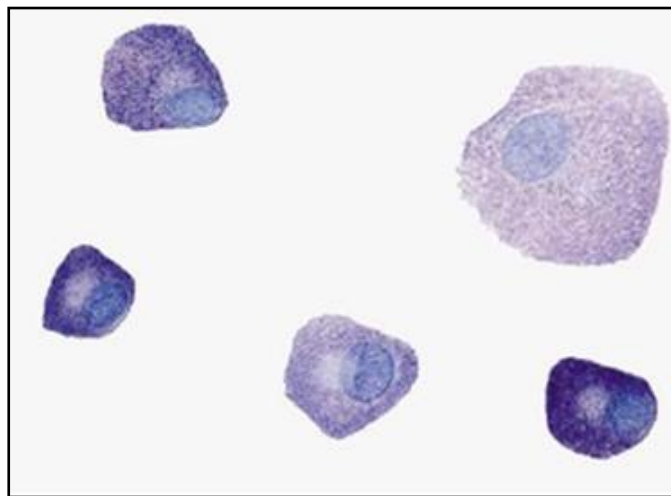


Figure 15: Toluidine blue staining of LAD2 cells. Magnification x200.

known MC markers and content histamine, tryptase and chymase. Indeed, 98% of LAD 2 cells are positive for tryptase and approximately 37% are positive for both tryptase and chymase. LAD 1 and 2 cells express surface FcεRI, KIT, and other markers including CD4, CD9, CD13, CD22, CD45, CD64, CD71, CD103, CD132, CD184 and CD195, and to a lesser degree, CD14, CD31 and CD32, whereas BM-derived MCs from the patient stained positive for CD13, KIT, but were negative for CD2, CD25, CD34 and CD35 (Kirshenbaum *et al.* 2003).

Compared to HMC-1 cells and its sublines, LAD 1 and 2 cells exhibit a more mature phenotype and closely resemble primary cultures of CD34+-derived human MCs, respond to rhSCF, and have functional FcεRI and FcγRI receptors (Kirshenbaum *et al.* 2003). Figure 15 shows toluidine blue staining of LAD2 cells. They can be stimulated to degranulate in an IgE-dependent manner (Gage *et al.* 2009). However, one major drawback of this cell line is its doubling time. Indeed, these cells double in numbers in approximately 3 weeks in the presence of 100 ng/ml of SCF. In addition, the cell culture only grows at high density. Thus, cell density should be maintained between 0.5×10^6 and 1×10^6 cells/ml and the cells should be splitted every 2–3 weeks. The addition of rhIL-3, rhIL-5, or rhIL-6 did not influence LAD cell numbers, while rhIL-4 reduced cell numbers. While HMC-1.2 cells harbor the *KIT* D816V activating mutation, LAD-1 and -2 cells do not exhibit *KIT* activating mutations, which may explain the requirement for SCF for the proliferation of these cells (Kirshenbaum *et al.* 2003).

LAD-2 cells may thus facilitate research in human MC survival and proliferation studies, as well as in MC activation through FcεRI and FcγRI, and have been distributed worldwide (Kirshenbaum *et al.* 2014). However, their slow doubling time, unstable phenotype in culture, and dependency on exogenous SCF for survival and proliferation, in addition to the difficulty to freeze and recover these cells, still limit their usefulness for particular applications such as high throughput screening of drugs.

1.9.3. LUVA cell line

The LUVA cell line is an immortalized human MC line grown from CD34+ enriched mononuclear cells (MNCs) derived from the PB of a donor with aspirin exacerbated respiratory disease, but with no

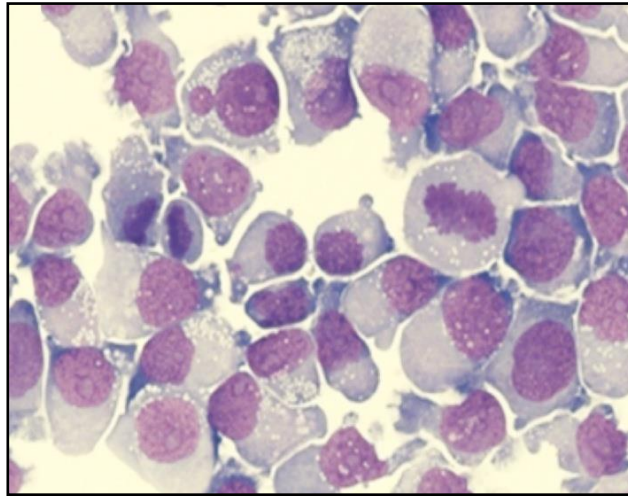


Figure 16: Morphological aspect of LUVA cells after May-Grünwald Giemsa (MGG) staining. Magnification x200.

clinical symptoms of mastocytosis, MC disorder or leukemia (Laidlaw *et al.* 2011). These cells proliferate without further addition of any cytokines or growth factors and they have no mutation in the *KIT* gene. They do not exhibit autophosphorylation of KIT and respond to rhSCF. In LUVA cells, KIT signaling pathway is functional, but not altered in terms of activity when compared to other MC lines (Laidlaw *et al.* 2011).

The LUVA cells have been published to resemble mature human MCs functionally and morphologically, to possess metachromatic cytoplasmic granules that are immunoreactive for tryptase, cathepsin G, and carboxypeptidase A3, to express surface FcεRI and to respond to IgE receptor cross-linkage leading to the release of β-hexosaminidase, PGD₂, thromboxane A₂ and macrophage inflammatory protein 1-β (Laidlaw *et al.* 2011). However, it has to be noticed that LUVA cells are behaving differently in long-term culture as compared to their description in the original publication. Indeed, in this publication, LUVA cells were presented as having high levels of expression of FcεRI, however, we and others have observed that these cells do not express surface FcεRI despite the very high mRNA expression for FcεRI. The reason for this discrepancy is unclear, but it is possible that this cell line tends to lose the expression of FcεRI upon long-term culture

Moreover, LUVA cells in our hands seem to be immature cells in term of granulation as no granules can be seen after MGG staining (Figure 16).

1.9.4. MCPV-1 cell lines

The human MCPV-1 (mast cell progenitor Vienna (1-4) cell lines were obtained in an attempt by Hoermann *et al.* to generate a cell line reflecting the biology of *RAS*-mutated advanced SM (Hoermann *et al.* 2014). Cord blood-derived MC progenitors were cultured in RPMI 1640 medium supplemented with 10% FCS, human SCF (100 ng/mL), and human IL-6 (100 ng/mL) for 8 weeks prior to lentiviral transduction with *HRAS* G12V, SV40 TAg, and *TERT* (Hahn *et al.* 1999). The human MCPV-1 was harvested and cloned by limiting dilution, generating four different clones, designated MCPV-1(1-4). Light microscopy of Wright-Giemsa-staining of MCPV-1 cells showed large, immature cells with bi-, tri-, or multilobated (often cloverleaf-like shaped) nuclei characteristic

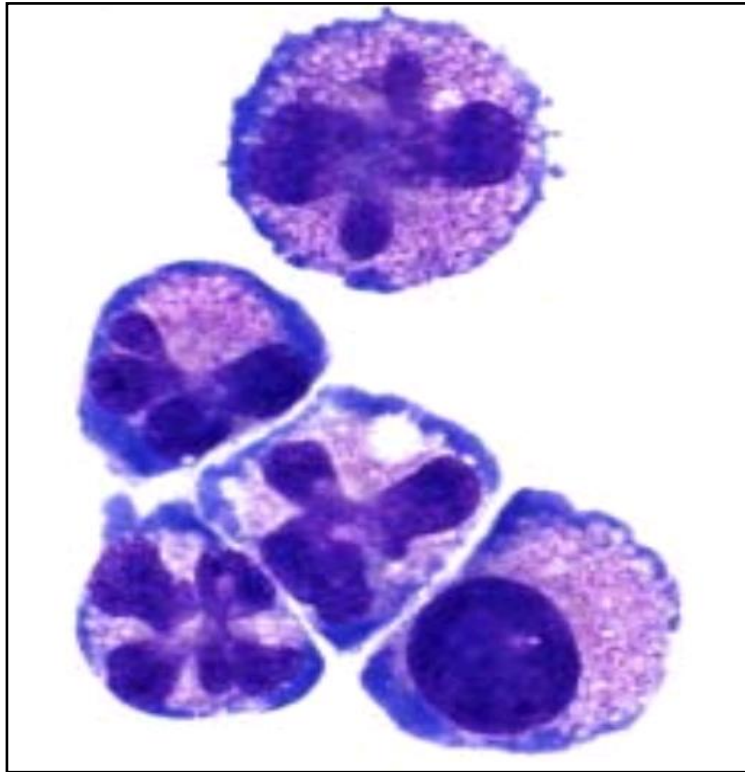


Figure 17: Morphological appearance of MCPV-1 cells after Wright-Giemsa staining. Magnification x500. (Figure adapted from Hoermann *et al.* 2014).

of MC precursors (Figure 17). The cells have abundant basophilic cytoplasm, cytoplasmic protrusions, and, in part, sparse granulations. Moreover, MCPV-1 cells exhibit an immunophenotype consistent with MC progenitors; they are positive for CD31, CD32, CD44, CD48, CD50, CD54, CD58, CD59, CD63, CD69, CD71, CD87, CD117, CD166 and CD203c. These cells are also positive for tryptase, and histidine decarboxylase (HDC) (Hoermann *et al.* 2014).

Interestingly, MCPV-1 cell line expresses the surface marker CD52, which has been recently identified as a molecular target in advanced forms of SM. This expression of CD52 in MCPV-1 cells appears to be driven through the activation of *RAS* and introduction of *RAS* mutant in the HMC-1 cells originally negative for CD52 leads to expression of CD52 on the membrane.

In addition, MAP kinase signaling is required for *RAS*-dependent expression of CD52 and MEK inhibitors were found to decrease such expression (Hoermann *et al.* 2014). Of note, in contrast to normal MCs, neoplastic MCs in patients with SM appear to consistently express the CD52 protein, as determined by immunohistochemistry (IHC). However, substantial surface expression of CD52 was detected exclusively in patients with advanced SM (Hoermann *et al.* 2014). A significant difference in CD52 mRNA and protein levels has also been recently documented between neoplastic MCs purified from patients with ISM and ASM (Teodosio *et al.* 2013). Moreover, alemtuzumab efficiently mediates lysis of CD52-positive neoplastic MCs *in vitro* (MCPV-1 cells) and *in vivo* in NSG mice injected intravenously (IV) with MCPV-1 cells (Hoermann *et al.* 2014).

2. Mastocytosis

2.1. Definition, epidemiology and diagnosis

Mastocytosis is a term used to denote a heterogeneous group of disorders characterized by the pathogenic and uncontrolled accumulation of MCs in one or more different tissues and organs including BM, skin, liver, spleen, lymph nodes and gastrointestinal (GI) tract (Brockow & Metcalfe 2001; Escribano *et al.* 2002; Valent *et al.* 2003; Horny *et al.* 2007; Metcalfe 2008; Amon *et al.* 2010;

Sokol *et al.* 2013). Mastocytosis is considered to be a rare disease amongst BM-derived hematologic neoplasms with an estimated prevalence of about 0.005-0.01% in Middle Europe or 1 per 10000 inhabitants (Van Doormaal 2013; Valent 2013; Brockow 2014). No apparent gender predominance has been reported and very few familial cases have been described. The clinical presentations of mastocytosis is heterogeneous, ranging from skin-limited disease (mainly Urticaria pigmentosa; UP) which occurs mainly in childhood and spontaneously regresses (Kettelhut & Metcalfe 1997; Brilley & Phillips 2008) to more aggressive subtype with extracutaneous manifestations which is generally seen in adults and may be associated with multiorgan failure and shortened survival (Lim *et al.* 2009; Sperr & Valent 2012; Sokol *et al.* 2013). Indeed, in childhood, the disease remains confined to the skin (Metcalfe 1991) and often occurs before the age of 2 years (Méni *al.* 2015), whereas in adulthood, almost all patients with UP-like skin lesions have systemic mastocytosis (SM), usually indolent SM (ISM) (Berezowska *et al.* 2013).

Patients with SM often show an elevated serum tryptase level (>20 ng/ml). The BM is almost always involved (Escribano *et al.* 1998; Honry *et al.* 2001; Valent *et al.* 2003; Akin & Metcalfe 2004; Honry *et al.* 2007; Arock & Valent 2010). However, the skin is often affected in SM (Pardanani 2012). Neoplastic MCs in SM typically carry activating point mutations in *KIT*, usually at codon 816 (Nagata *et al.* 1995; Longley *et al.* 1996; Longley *et al.* 1999; Fritsche-polanz *et al.* 2001; Féger *et al.* 2002), have an abnormal morphology with spindle-shaped appearance and are hypogranulated, show an aberrant expression of CD2 and/or CD25 and form compact infiltrates in extracutaneous tissues. By contrast, in cutaneous disease found in children, a mutation of codon 816 (exon 17) is found in 42% of cases, and mutations outside exon 17 are observed in 44% of the cases, half of these mutations being located in the fifth Ig loop of *KIT*'s extracellular domain encoded by exons 8 and 9 (Bodemer *et al.* 2010). Interestingly, in adults, SM is sometimes (5 to 20% of the cases, depending on the studies) associated with another hematological non MC diseases (SM-AHNMD) (Arock *et al.* 2015).

The incidence of the prognostically unfavorable subtypes of SM including the aggressive SM (ASM) and mast cell leukemia (MCL) is low compared to that of indolent forms of SM (ISM). ASM and MCL usually present without skin lesions but often mimic more common hematologic neoplasms like

Table VI: The WHO criteria to diagnose SM (Valent *et al.* 2001; Valent *et al.* 2001; Horny *et al.* 2008).

The WHO criteria to diagnose SM	
Major	Multifocal dense infiltrates of MCs (≥ 15 MCs in aggregates) in BM and/or other extracutaneous organs, confirmed by special stains such as MC tryptase.
Minor	In MC infiltrates in BM or other extracutaneous organs, $>25\%$ of MCs are spindle-shaped or have atypical morphology (cytoplasmic projections, hypogranulation); or in BM smears, $>25\%$ of MCs are spindle shaped or otherwise atypical
	Activating point mutation at codon 816 of <i>KIT</i> is detected in BM, PB, or other extracutaneous organs
	MCs in BM, PB, or other extracutaneous organs express CD2 and/or CD25 in addition to normal MC markers as determined by flow cytometry
	Serum tryptase level persistently exceeds 20 ng/mL (does not count in patients who have an associated clonal hematological non-mast-cell disease; AHNMD)

Table VII: The B-“Borderline Benign“ findings and C-“Consider Cytoreduction“ findings useful to assess MC burden and aggressiveness of SM (Valent *et al.* 2001; Valent *et al.* 2003; Arock *et al.* 2015).

“B” findings (borderline-benign)
1. BM biopsy showing $>30\%$ of MCs infiltration and/or serum tryptase level exceeds 200 ng/mL
2. Signs of dysplasia or myeloproliferation, in non-MC lineage(s), but insufficient criteria for definitive diagnosis of a hematopoietic neoplasm (AHNMD), with normal or slightly abnormal blood counts.
3. Hepatomegaly without impairment of liver function, and/or palpable splenomegaly without hypersplenism, and/or lymphadenopathy on palpation or imaging.
“C” findings (consider cytoreduction)
1. BM dysfunction manifested by one or more cytopenia(s) (absolute neutrophil count $ANC < 10^9/L$, hemoglobin $Hgb < 10$ g/dL, or platelets $< 100 \times 10^9 /dL$), but no obvious non-MC hematopoietic malignancy
2. Palpable hepatomegaly with impairment of liver function, ascites, and/or portal hypertension
3. Skeletal involvement with large osteolytic lesions and/or pathological fractures
4. Palpable splenomegaly with hypersplenism
5. Malabsorption with weight loss due to gastrointestinal MC infiltrates

malignant lymphoma or myeloid leukemia at first presentation.

The diagnosis of mastocytosis may be challenging to establish, especially for those patients who present with features of myeloid or lymphatic tumors and do not have cutaneous involvement (Parker 1991; Valent *et al.* 2007). For each patient with suspected mastocytosis, a clinical workup should be performed including information about allergic reactions and skin rashes, and serum typtase level. In patients with a rash and elevated serum typtase level, histologic investigation of the BM must be performed (Valent *et al.* 2013). Precise defining criteria to diagnose SM have been published by the World Health Organization (WHO) (Valent *et al.* 2001; Horny *et al.* 2008). These WHO criteria to diagnose SM are presented in table VI (Valent *et al.* 2001; Valent *et al.* 2001; Horny *et al.* 2008). The diagnosis of SM is established when one major criterion and one minor criterion or at least three minor criteria are present. Once the diagnosis is established, patients with SM are categorized further according to the presence of B “Borderline Benign“ findings reflecting the disease burden and C “Consider Cytoreduction“ findings reflecting the disease aggressiveness (Table VII) (Valent *et al.* 2001; Valent *et al.* 2003; Arock *et al.* 2015). Patients who have SM without B- or C-findings and without signs of an AHNMD are diagnosed as indolent SM (ISM). Patients with two or more B-findings, but no C-findings are diagnosed with smoldering SM (SSM). Finally, patients who have one or more C-findings are candidates for therapeutic cytoreduction and are considered as aggressive SM (ASM) or MC leukemia (MCL) (Valent 2013; Arock *et al.* 2015). MCL is excluded in patients with ASM when the MCs in BM smears represent less than 20% of total nucleated cells, whereas patients with at least 20% of MCs in BM are diagnosed with MCL (Valent 2013).

KIT mutations in exon 17 at codon 816 can be detected in PB, BM aspirate, or other lesional tissue samples, and the presence of *KIT* D816V is one of minor criteria for the diagnosis of SM. This most common mutation arising from substitution of valine for aspartate is detected in neoplastic MCs of more than 95% of adult patients with SM and in 42% of pediatric patients with cutaneous mastocytosis (CM) (Longley *et al.* 1996; Longley *et al.* 1999; Orfao *et al.* 2007; Bodemer *et al.* 2010; Cardet *et al.* 2013). Because this mutation results in a conformational change in the KD of *KIT* that renders it resistant to imatinib, the TK inhibitor usually effective in GI stromal tumors (GISTs) and chronic

Table VIII: Variants and subvariants of mastocytosis according to the 2008 WHO classification (Table adapted from Horny *et al.* 2008; Arock & Valent 2010).

Variant (Abbreviation)	Subvariants	Diagnostic criteria	Prognosis
Cutaneous mastocytosis (CM)	Urticaria pigmentosa (UP)= Maculopapular CM (MPCM) Diffuse CM (DCM) Mastocytoma of skin	No systemic involvement (most patients are children)	Good
Indolent systemic mastocytosis (ISM)	Smouldering systemic mastocytosis (SSM) Isolated bone marrow mastocytosis Well-differentiated systemic mastocytosis (WDSM)	No C findings Mostly seen in adults	Good
Systemic mastocytosis with an associated clonal hematologic non-MC lineage disease (SM-AHNMD)	<ul style="list-style-type: none"> • Systemic mastocytosis with acute myeloid leukemia (SM-AML) • Systemic mastocytosis with myelodysplastic syndrome (SM-MDS) • Systemic mastocytosis with myeloproliferative neoplasm (SM-MPN) • Systemic mastocytosis with eosinophilic leukemia SM-CEL • Systemic mastocytosis with chronic myelomonocytic leukemia (SM-CMML) • Systemic mastocytosis with non-hodgkin's lymphoma (SM-NHL) • SM-myeloma 	Appropriate WHO criteria have to be applied in all patients in order to diagnose and classify AHNMD	Variable depending on AHNMD
Aggressive systemic mastocytosis (ASM)	Lymphadenopathic SM with eosinophilia	Existence of signs indicating organ failure secondary to mast cell infiltration (C findings): <ul style="list-style-type: none"> • Bone marrow failure (marked cytopenia) • Hepatic failure with ascites • Splenomegaly with hypersplenism • Osteolysis and pathological fractures • Digestive tract involvement with malabsorption and weight loss 	Poor
Mast cell leukemia (MCL)	Aleukemic MCL	Atypical MCs (multilobulated nucleus, multinucleated cells) and >20% of MC found in bone marrow smears	Very poor
Mast cell sarcoma (MCS)		Malignant tumor destroying the soft tissue Atypical MCs	Very poor
Extracutaneous mastocytoma (ECM)		Rare benign tumor Normal mature cells	Good

myeloid leukemia (CML), most patients with SM do not respond to imatinib therapy (Ma *et al.* 2002; Zermati *et al.* 2003; Akin *et al.* 2003).

2.2. Classification of mastocytosis

The pathological accumulation of MCs in mastocytosis and the release of their mediators are responsible for the clinical presentations and symptoms including pruritus, flushing, nausea, diarrhea and vascular instability. In addition, inSM severe symptoms like osteoporosis, fractures, and even organ failure can occur. This complex pattern of symptoms and their severity lead to a classification listed in 2008 by the World Health Organization (WHO) and subsequent prognosis for each case of mastocytosis (Horny *et al.* 2007; Horny *et al.* 2008; Arock & Valent 2010) (Table VIII).

2.2.1. Cutaneous Mastocytosis (CM)

MC infiltrates in CM are restricted to the skin and variants of CM are defined by their clinical presentation. Patients with CM show a wide variety of skin alterations (Rueff *et al.* 2006). Even though all skin lesions in CM are due to an abnormally elevated MC number in the dermis, the number and morphology of efflorescences may vary from patients to patient (Longley *et al.* 1995).

In addition, while there is no systemic infiltration and the disease is limited to the skin, it can be associated with systemic symptoms due to the release of MC mediators (Bodemer *et al.* 2010).

The most common subvariant of CM is termed UP and presents as disseminated macular or maculopapular rash. UP is characterized by symmetrically distributed 0.5 cm red-brown macules and papules, which induce erythema, wheal and pruritus, the so-called Darrier's sign, when rubbed or scratched (Wolff *et al.* 2001; Hartmann & Henz 2001). Patient with CM often exhibit the brownish-red maculopapular lesions of UP. In UP, MCs are either loosely scattered, with a marked tendency to aggregate around blood vessels and adnexae in the upper dermis, or form small compact infiltrates. Larger compact transdermal infiltrates of MCs are seen in nodular variant of UP and solitary mastocytoma of the skin. Diffuse CM is less frequently diagnosed. The solitary localized mastocytoma (of the skin) is also rare (Kanwar & Dhar 1993), and has a benign clinical course. Thus, most MC

tumors of the skin appear to be benign. Intradermal MCs always coexpress tryptase and CD117, while only few cells in some cases express CD25 (Berezowska *et al.* 2013).

2.2.2. Indolent systemic mastocytosis (ISM)

Indolent systemic mastocytosis (ISM) is the most common variant of SM and involves the BM, usually with limited increase of MC burden. Most of the patients with ISM have skin involvement, usually UP, and some patients may present with other organ involvement such as the GI tract, lymph node, spleen or liver, but without organ dysfunction (Valent *et al.* 2001). In general, the MC burden in typical ISM does not exceed 30% in BM biopsy and a few percent in BM smears. The shift into an aggressive variant or development of an associated malignancy is possible but not frequent in these patients. It is important to know that almost all patients (>95%) with adult-onset UP-like skin lesions might have ISM, hence the BM biopsy is recommended for such patients (Ridell *et al.* 1986; Czarnetzki *et al.* 1988; Valent 2013). ISM shows a prolonged clinical course in almost all patients with excellent prognosis, nearly normal life expectancy, and survival of two decades and more (Pardanani *et al.* 2009; Escribano *et al.* 2009; Pardanani *et al.* 2010). Serum tryptase level is raised in comparison to CM with levels ranging from 20 to 200 ng/ml. A rare subvariant of ISM is the so-called smoldering systemic mastocytosis (SSM) (Valent *et al.* 2002) with pronounced MC infiltration (>30% in BM biopsy), organomegaly and tryptase levels above 200 ng/ml. Smoldering systemic mastocytosis (SSM) is defined by the presence of 2 or more “B-findings” (Table VII) (Pardanani *et al.* 2010). The life expectancy of patient with SSM is variable (Valent *et al.* 2002). Whatsoever, *KIT* D816V is the most frequent abnormality found in >80% of all ISM patients and can be detected by sensitive quantitative polymerase chain reaction techniques (Garcia-Montero *et al.* 2006; Kristensen *et al.* 2011). More recently, circulating non-MCs elements harboring the *KIT* D816V mutation have been detected in PB and found to be characteristic of ISM (Garcia-Montero *et al.* 2006). In addition, it has been shown that allele burden of the *KIT* D816V mutation in PB predicts for the risk of progression from ISM to ASM (Valent 2013; Kirstensen *et al.* 2014). Besides, another subvariant, termed “well-differentiated systemic mastocytosis” (WDSM) is characterized by BM multifocal infiltrates by round,

mature CD25 MCs and by the absence of mutation of 816 codon of *KIT* or of other *KIT* mutations (Teodosio *et al.* 2010)

2.2.3. Systemic mastocytosis with an associated clonal hematologic non mast cell lineage disease (SM-AHNMD)

Systemic mastocytosis (SM) is found in 5-20% of cases associated with another hematologic malignancy, and is thus termed SM with an associated hematologic non-MC lineage disease (SM-AHNMD). The AHNMD can be a myelodysplastic /myeloproliferative syndrome, a myeloid leukemia or a myelodysplastic syndrome (Table VIII) (Horny *et al.* 2008; Stoecker & Wang 2012), while lymphoproliferative neoplasms are much less commonly implicated (Sperr *et al.* 2000; Pardanani *et al.* 2009; Sotlar *et al.* 2010).

The *KIT* D816V mutation is identified in >70% of the patients with SM-AHNMD (Sotlar *et al.* 2010). However, in a subset of patients with SM-CEL (SM with chronic eosinophilic leukemia), *FIP1L1/PDGFR*A mutant, but no *KIT* D816V mutation, is found (Valent 2013). In addition, none of the rare SM patients with lymphoproliferative AHNMDs presented the *KIT* D816V mutation (Sotlar *et al.* 2010).

The prognosis of patients with SM-AHNMD is dictated by the course and progression of AHNMD (Pardanani *et al.* 2009; Arock & Valent 2010). Since the AHNMD may obscure the SM compartment, SM-AHNMD is a primarily morphological diagnosis based on a thorough investigation of BM trephine specimens including tryptase and CD25 immunohistochemistry (Horny *et al.* 2004).

2.2.4. Aggressive systemic mastocytosis (ASM)

As mentioned above, ISM can develop into an aggressive form with progressive infiltration of immature and atypical MCs and subsequent organ destruction and failure ('C findings') (Valent 1996; Garcia-Montero *et al.* 2006). ASM is much less common than ISM, representing only about 5% of patients with SM, and follows an accelerated course (Valent *et al.* 2000; Valent *et al.* 2003). However, in a subgroup of patients, a slower progression is seen (Valent *et al.* 2001). The life expectancy of

patients with ASM is poor. Patients with ASM share common clinical features with SM-AHNMD patients and MCL patients in that they are more likely to be older, present with end-organ damage (organopathy), resulting from massive MC infiltration and usually without UP-like skin lesions, invariably elevated serum tryptase levels reflecting a significant MC burden, and activating *KIT* mutations.

In ASM, the criteria to diagnose SM are fulfilled and MCL and AHNMD are excluded after BM and PB examinations. The histology of the BM in ASM shows a variable degree of infiltration. MCs usually comprise less than 20% of nucleated cells in BM smears, are tryptase+ and KIT (CD117) + and in a majority of cases express CD2 and/or CD25. In addition, they do express aberrantly CD30 (Valent *et al.* 2011). In ASM, malignant MCs are hypogranulated and show atypical and high-grade morphology with bi- or multilobed nuclei (Valent *et al.* 2001). Moreover, if most patients with ASM have *KIT* D816V mutation, other *KIT* mutations such as V559I and D820G have been also found (Pignon *et al.* 1997; Nakagomi & Hirota 2007).

Contrasting to ISM and SSM, C findings are detectable in ASM as a sign of organopathy due to infiltration by neoplastic MCs. In particular, patients show abnormal myelopoiesis with significant blood count abnormalities, hepatomegaly with impairment of liver function due to MC infiltration (often with ascites), large osteolyses (sometimes with pathologic fractures), malabsorption with weight loss due to GI-tract infiltration, splenomegaly with hypersplenism, or life-threatening impairment of organ function in other organ systems. The most commonly affected organs are liver, BM, spleen, and the GI tract (Horny *et al.* 2007; Valent *et al.* 2011).

Abnormalities in blood counts may be cytopenia(s), leukocytosis, monocytosis, eosinophilia, basophilia, or thrombocytosis. However, no circulating MCs are detectable (Valent *et al.* 2001). A distinct and rare subvariant of ASM with prominent eosinophilia in blood and tissues and generalized lymphadenopathy (clinically mimicking malignant lymphoma) has been described as lymphadenopathic mastocytosis with eosinophilia (Hauswirth *et al.* 2002).

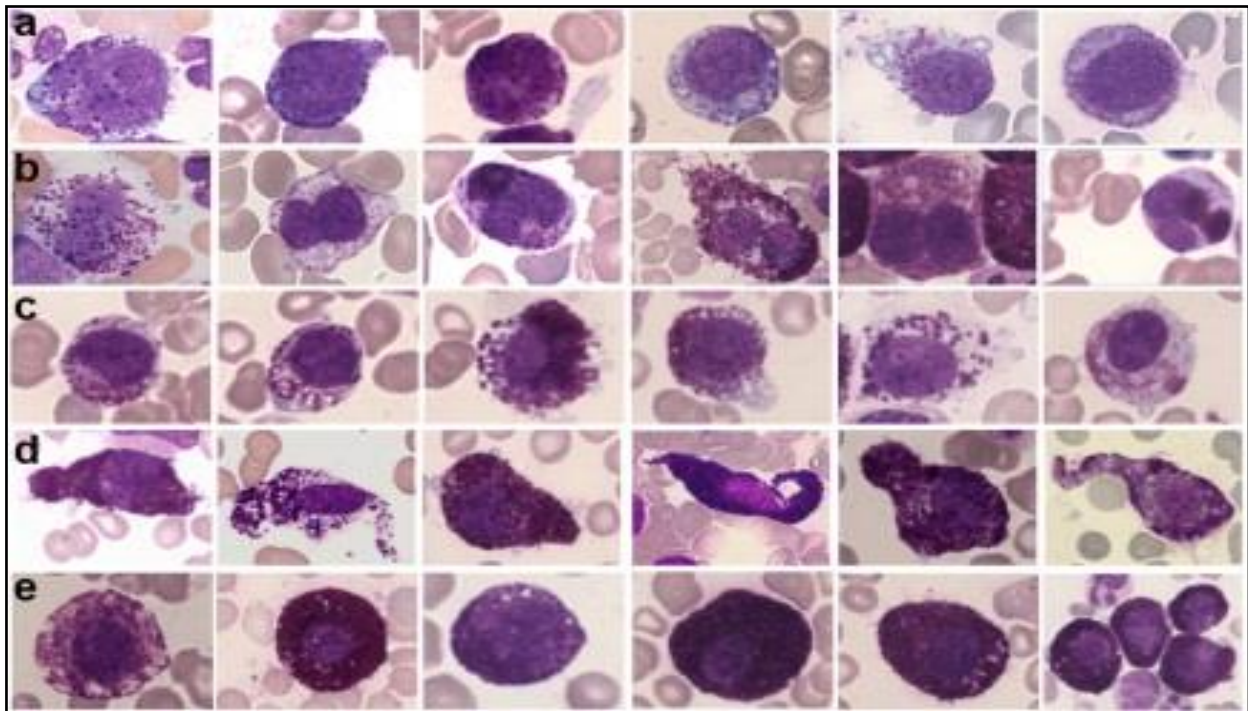


Figure 18: Different morphological aspects of leukemic MCs circulating in the PB of patient with MCL after May-Grünwald Giemsa (MGG) staining. (a) Metachromatic granulated blast-like MCs. (b) Atypical MCs type II also known as promastocytes. (c) More immature atypical MCs type I. (d) More mature atypical MCs type I; spindle-shaped MCs. (e) Mature round MCs. Magnification: x500.

2.2.5. Mast cell leukemia (MCL)

Mast cell leukemia (MCL) is a rare and highly aggressive subvariant of mastocytosis involving less than 0.5% of all patients with mastocytosis in the database of the French Reference Center for Mastocytosis (CEREMAST) (Georgin-Lavialle *et al.* 2013). MCL is characterized by leukemic infiltration of various organs by highly atypical MCs (Figure 18), including the BM (Valent *et al.* 2003), by progressive organopathy including BM failure from MC infiltration, and by the presence of a considerable number of atypical MCs on the BM smear (MC numbers in BM smears must exceed 20% of all nucleated cells in smears). Circulating MCs are found in most cases with MCL (more than 10% of blood cells in typical MCL), while aleukemic variants of MCL are rare in which no circulating MCs are seen (Horny *et al.* 2002).

MCL shares several clinicobiologic aspects with ASM (Valentini *et al.* 2008). Hepatosplenomegaly, anemia, and thrombocytopenia are usually present and serum tryptase level is usually high. MCs in MCL are usually CD2 and/or CD25 positive. However, the positive coexpression of CD2 and CD25 was found to be very frequent among MCL patients who are *KIT* D816V positive (66%) but not in those who are *KIT* D816V negative (25%) (Georgin-Lavialle *et al.* 2013). However, one-third of MCL cases reported in the literature have a double-negative CD2/CD25 immunophenotype (Valent 2002; Horny *et al.* 2007; Arredondo *et al.* 2010).

Although the *KIT* D816V mutation has been described in the literature as the most frequently encountered *KIT* mutation in patients with SM, this is not the case in MCL patients (Joris *et al.* 2012). Indeed, *KIT* WT or exon 9 to 13 *KIT* mutations are more commonly found in MCL and thus complete *KIT* gene sequencing is needed (Akin *et al.* 2004; Georgin-Lavialle *et al.* 2012; Georgin-Lavialle *et al.* 2013). Georgin-Lavialle *et al.* reported that death occurs more frequently in the MCL patient without D816V mutation (69%) than for those with the D816V mutation (46%), although the median survival time does not differ (Georgin-Lavialle *et al.* 2013).

MCL can appear *de novo* or after previous mastocytosis, and is thus termed “secondary MCL” (Georgin-Lavialle *et al.* 2013; Valent *et al.* 2014). The clinical features including hepatosplenomegaly, lymph node enlargement, ascites, and symptoms of MC activation are comparable between these 2

groups. However, patients with secondary MCL present more frequent cutaneous manifestations (UP), and no gastroduodenal ulcers were found, while gastroduodenal ulcers were found in up to 38% of the *de novo* MCL cases. Besides, in the *de novo* MCL, pathologic MCs are highly atypical, proliferate rapidly, infiltrate all organs (including the GI tract), and degranulate easily, releasing large amounts of histamine, leading to gastroduodenal ulcer formation (Georgin-Lavialle *et al.* 2013). Furthermore, it has been recently proposed that MCL can be divided into acute MCL and chronic MCL, based on the presence or absence of C findings (Valent *et al.* 2014).

MCL is rarely associated with clonal hematologic non-MC disease (Valent *et al.* 2010), but the prognosis of these few patients is always poor. Only 4 cases were reported in the literature: three cases of AHNMD were myelodysplastic syndromes and one case was chronic myelomonocytic leukemia. Of note, AHNMD was only found in the MCL patients with *KIT* D816V mutation.

All in all, the prognosis of patients with MCL is very poor (Valent *et al.* 2001; Sperr & Valent 2012). Indeed, therapy usually fails and the mean survival time is only of 6.6 months (range 2-14 months) (Noack *et al.* 2004).

2.2.6. Mast cell sarcoma (MCS)

Mast cell sarcoma (MCS) is an exceedingly rare clinicopathologic variant of mastocytosis characterized by a unifocal accumulation of highly atypical neoplastic MCs that grow in a locally destructive manner. MCS has been documented in at least 6 human cases in the literature (Horny *et al.* 1986; Kojima *et al.* 1999; Guenther *et al.* 2001; Valent *et al.* 2001; Bautista-Quach *et al.* 2013; Ryan *et al.* 2013).

It is noteworthy that most reported MCSs occur in tissues not commonly involved by SM and the organs involved are different from BM or skin (larynx, colon, meningeal site, intracranial site) (Horny *et al.* 1986; Kojima *et al.* 1999; Valent *et al.* 2001; Horny *et al.* 2007). Furthermore, the cell atypia in MCS may be similar to that seen during MCL. In fact, MCs in MCS display a high-grade cytology and exhibit bi- or polylobed nuclei. They are rather immature (often blast like) with a hypogranulated cytoplasm, and a high nucleus/cytoplasm ratio. Since cytomorphological atypia of this rare entity

(MCS) is usually very high (according to a grade 3 sarcoma), it is impossible to achieve the correct diagnosis without appropriate immunohistochemical stainings, particularly in differentiating this lesion from other neoplasms that are more likely to occur in infancy. Of note, neoplastic MCs in MCS are tryptase+ and KIT+ (Valent *et al.* 2001).

Finally, the prognosis of patients with MCS is poor. However, patients receiving chemotherapy and radiation may survive a few years, although MCS can progress to secondary MCL (Valent *et al.* 2001; Pardanani 2013).

2.2.7. Extracutaneous mastocytoma (ECM)

Extracutaneous mastocytoma (ECM) is a localized benign tumor characterized by a unifocal MC tumor without no aggressive or destructive growth pattern and no evidence of SM (Valent *et al.* 2001; Castells 2006; Horny *et al.* 2007; Arock *et al.* 2015). ECM is extremely rare and has been found almost exclusively in the lung (Scherwin *et al.* 1965; Charrette *et al.* 1966; Kudo *et al.* 1988; Mylanus *et al.* 2000; Valent *et al.* 2001). In pulmonary ECM, the clinical features are those of an intrathoracic tumor with no specific macroscopic or clinical signs. In contrast to MCS, MCs in ECM show a low-grade cytology and exhibit relatively monomorphic strongly metachromatic round mature phenotype.

The prognosis for patients with ECM is good (Scherwin *et al.* 1965; Charrette *et al.* 1966; Kudo *et al.* 1988; Mylanus *et al.* 2000; Valent *et al.* 2001) and no progression to ASM or MCL occurs (Valent *et al.* 2001).

2.3. Clinical features of mastocytosis

Mastocytosis presents with a wide variety of clinical manifestations ranging from asymptomatic disease to a highly aggressive course with multi-system involvement. The clinical symptoms are related to the excessive release of MC mediators and/or to MC burden and can be observed in all categories of mastocytosis. Typical skin symptoms include pruritus, UP and flushing (Sperr & Valent 2012). In patients with ISM, symptoms are related to local or remote effects of excess mediator release from MCs, either spontaneously or in response to triggering stimuli (Castells & Austen 2002; Castells

Table IX: MC mediators and the clinical mediator-related symptoms of mastocytosis (Table adapted from Arock *et al.* 2015).

Clinical symptoms	MC mediator(s) potentially involved
Headache	Histamine, prostaglandine D2 (PGD2)
Hypotension, vascular instability	Histamine, PGD2, platelet activating factor (PAF), leukotrienes (LTs)
Arrhythmia	PAF, tumor necrosis factor (TNF α)
Anaphylactic shock	Histamine, PGD2, LTs, PAF
Increased vascular permeability	Histamine, vascular endothelial growth factor (VEGF), LTs, PAF
Increased local angiogenesis	Oncostatin M (OSM), VEGF
Tissue fibrosis	Transforming growth factor beta (TGF β), OSM, fibroblast growth factor (FGF)
Eosinophilia	Interleukins IL-3, IL-5
Leukocyte recruitment and activation	Histamine, PAF, cytokines, chemokines
Anticoagulation, bleeding	Heparin, tryptase
Fibrinolysis, bleeding	Tissue plasminogen activator (tPA), heparin
Fibrinogenolysis	Tryptase
Skin pruritus	IL-31, histamine,
Urticaria	Histamine, PAF, LTs
Edema	Histamine, LTs, VEGF, cytokines
Bronchoconstriction	Histamine, PAF, LTs, PGD2, endothelin
Pulmonary edema	Histamine, PAF, LTs
Gastric hypersecretion	Histamine, LTs
Cramping, abdominal pain	Histamine, PAF, LTs
Diarrhea	Histamine
Bone remodeling	IL-6, tryptase, FGF, receptor activator of NF- κ B ligand (RANKL), chymotryptic proteases
Osteoporosis	Proteases, Heparin

2006). Exogenous and endogenous triggers, such as temperature changes, infections, physical exercise, consumption of alcohol, nonsteroidal anti-inflammatory drugs (NSAIDs) and/or emotional stress are variable from one patient to another. In addition, patients present a variable and often changing pattern of symptoms. Other mediator-related symptoms include nausea, vomiting, diarrhea, headache, dizziness, hypotension, gastroduodenal ulcers, tissue fibrosis, breathing difficulties, abdominal pain, edema, arthralgia myalgia, and arrhythmia. MC mediators involved in the most prominent clinical symptoms of mastocytosis are listed in Table IX. Of note, not all patients experience all these symptoms, and symptoms occur as attacks that may be brief or prolonged, but the duration is usually in the range of 15 to 30 minutes. In addition, symptoms may present in some patients as anaphylactic reaction, which might be life-threatening (anaphylactic shock) (Akin & Metcalfe 2004; Butterfield 2006). The prevalence of anaphylaxis has been reported to be 20% to 56% in adult patients with various forms of mastocytosis (Florian *et al.* 2005; González de Olano *et al.* 2007; Brockow *et al.* 2008). Nevertheless, not all SM patients bearing *KIT* D816V mutation suffer from anaphylaxis. Therefore, other genetic polymorphisms or mutations in MC signaling components, apart activating *KIT* D816V mutation, contribute to dysregulation and predispose to anaphylaxis (Metcalfe & Akin 2001; Brown *et al.* 2008). Furthermore, osteopenia/osteoporosis has long been recognized in SM patients. Thus, Routine measurements of bone mineral density and vertebral morphometry are warranted (Barete *et al.* 2010; Rossini *et al.* 2011; Rossini *et al.* 2014). Less commonly, osteosclerosis is also seen, and in some patients both osteoporosis and osteosclerosis may be observed in different sites (Johansson *et al.* 1996).

In addition, in adult mastocytosis, neurologic symptoms including lack of concentration, irritability, anxiety and/or depression are frequent and the pathophysiology of these symptoms in a majority of cases is not known but could be linked to tissue MC infiltration, MC mediator release, or both (Moura *et al.* 2014). Patients with systemic mastocytosis (SM, discussed later) also complain from “brain fog symptoms” which include the loss of attention, focus, short term memory and ability to multitask (Moura *et al.* 2012; Jennings *et al.* 2014), headaches, and autism spectrum disorders (ASDs) (Theoharides *et al.* 2015).

By contrast to ISM, it may be difficult to attribute symptoms to mastocytosis in patients with SM-AHNMD, as the patients may have signs and symptoms related to the AHNMD. Moreover, in advanced SM such as in ASM and MCL, destructive infiltration of neoplastic MCs is responsible for the symptoms related to end-organ damage including malabsorption and weight loss, osteolyses and pathologic bone fractures, hypersplenism, hepatomegaly with impairment of liver function often with ascites, significant cytopenia(s) (Valent *et al.* 2001; Valent *et al.* 2003; Valent *et al.* 2005; Pardanani *et al.* 2006; Robyn & Metcalfe 2006; Lim *et al.* 2009; Valent *et al.* 2010). These symptoms represent so-called “C findings” and found in patients with ASM and patients with MCL (Valent *et al.* 2001; Valent *et al.* 2003; Valent *et al.* 2014).

MCs are also involved in MC activation syndrome (MCAS) which distinguishes a group of patients clinically presenting with recurrent episodes of anaphylaxis, no skin lesions, and only one or two minor diagnostic criteria for systemic mastocytosis (SM) but lacking the major criterion, i.e. compact infiltrates of MCs in BM. In MCAS, MCs may display cytomorphological atypia, aberrant expression of CD25 or the presence of *KIT* D816V mutant, but all three features (which would be sufficient for the diagnosis of SM) are not detectable (Horny *et al.* 2007; Valent *et al.* 2012). MCA can occur in any variant of mastocytosis. In such cases, the presence of MCA(S) should be recorded as such or by the subscript ‘SY’, for ‘symptoms’ (e.g. SM_{SY}) (Valent *et al.* 2001; Valent *et al.* 2007; Valent *et al.* 2012).

2.4. Pathophysiology of mastocytosis

During the past years, the scientific knowledge about the pathophysiology of mastocytosis has been rapidly developed. Since SCF was identified as the major cytokine responsible for MC proliferation, increased secretion of SCF was hypothesized to be involved in the pathophysiology of mastocytosis, but no data confirmed this hypothesis. This lack of SCF deregulation led the scientists to search for abnormalities in the SCF receptor, KIT. The presence of such abnormalities (point mutations) in *KIT* was firstly found in the HMC-1 cell line (Furistu *et al.* 1993), and then confirmed in patient’s pathological samples. Thus, it is well known today that most cases of mastocytosis are associated with an activating *KIT* defect. Interestingly, all the various *KIT* mutations found in different variants of

Table X: Molecular somatic lesions and abnormalities found in patients with mastocytosis. CM, cutaneous mastocytosis; SM, systemic mastocytosis; SM-AHNMD, SM with an associated haematologic clonal non mast cell lineage disease; CEL, chronic eosinophilic leukemia; AML, acute myeloid leukemia; PMF, primary myelofibrosis (Table adapted from Valent 2013).

Molecular abnormality	Estimated frequency in patients with SM	Reported in patients with
<i>KIT</i> D816V	>80%	all SM variants, rarely in CM
<i>KIT</i> D816Y	<5%	CM, ISM, SM-AHNMD
<i>KIT</i> D816F	<5%	CM
<i>KIT</i> D816H	<5%	MCL, ASM, SM-AHNMD
<i>KIT</i> D820G	<5%	ASM
<i>KIT</i> V560G	<5%	ISM
<i>KIT</i> F522C	<5%	ISM
<i>KIT</i> E839K	<5%	CM
<i>KIT</i> V530I	<5%	SM-AHNMD
<i>KIT</i> K509I	<5%	SM (familial type)
Other <i>KIT</i>	<5%	CM and/or SM variants
<i>FIP1L1/PDGFR</i> A	<5%	SM-CEL
<i>AML1/ETO</i>	<5%	SM-AML with t(8;21)
<i>JAK2</i> V617F	<5%	SM-PMF
RAS mutations	<5%	ASM, SM-AHNMD
TET2 mutations	<5%	SM-AHNMD, ISM, ASM
<i>DNMT3A</i>	<5%	ISM, SM-AHNMD
ASXL1 mutations	<5%	SM-AHNMD
CBL mutations	<5%	SM-AHNMD

mastocytosis result in constitutive autophosphorylation of the KIT molecule, independently from its ligand (SCF), thereby leading to an enhanced differentiation, survival and activation of MCs (Tsujiura *et al.* 1994; Akin & Metcalfe 2004; Orafo *et al.* 2007; Metcalfe 2008).

As mentioned previously, the finding of activating point mutation arising from substitution of valine for aspartate at codon 816 of *KIT* is the molecular hallmark of mastocytosis. In 1995, Nagata *et al.* identified *KIT D816V* mutation in the PB of four patients with mastocytosis (Nagata *et al.* 1995). This mutation is located in the phosphotransferase domain (PTD) of the receptor and detected in neoplastic MCs of majority of adult patients with SM regardless the variant of SM and in approximately 40% of skin biopsies from pediatric patients with CM (Longley *et al.* 1996; Longley *et al.* 1999; Orafo *et al.* 2007; Bodemer *et al.* 2010; Cardet *et al.* 2013; Arock *et al.* 2015). Zappulla *et al.* showed that in a model of transgenic mouse for human *KIT D816V*, MC hyperplasia was observed along with the expression of the transgene in these cells, indicating the involvement of *KIT* defects in the pathophysiology of SM (Zappulla *et al.* 2005). Whereas the *KIT D816V* mutant is thought to play an important role in ISM that presents with MC mediator-related symptoms and have no signs of a substantial MC proliferation, in contrast the *KIT D816V* mutation alone is not believed to be responsible for the major manifestations and aggressiveness of SM-AHNMD, ASM and MCL (Valent *et al.* 1999; Tefferi & Pardanani 2004).

Indeed, in these patients, additional genetic defects have been identified including other *KIT* mutations apart from D816V (Pignon *et al.* 1997; Pullarkat *et al.* 2000; Akin *et al.* 2004; Nakagomi & Hirota 2007), *RAS* mutations (Wilson *et al.* 2011), *TET2* mutations (Tefferi *et al.* 2009; Traina *et al.* 2012; Soucie *et al.* 2012), *ASXL1* (additional sex combs-like 1) (Trania *et al.* 2012), *SRSF2*, *ASXL1*, *SRSF2* (Hanssens *et al.* 2014) or *RUNX1* and mutations in IgE receptor genes (Spector *et al.* 2012). These defects might decisively contribute to the clinical heterogeneity and the aggressiveness of SM and may represent new therapeutic targets (Arock *et al.* 2015). Interestingly, overall survival was found significantly shorter in patients with additional aberrations (Schwaab *et al.* 2013). Table X summarizes the molecular somatic lesions and abnormalities found in patients with mastocytosis and their estimated frequency in patients with SM (Valent 2013).

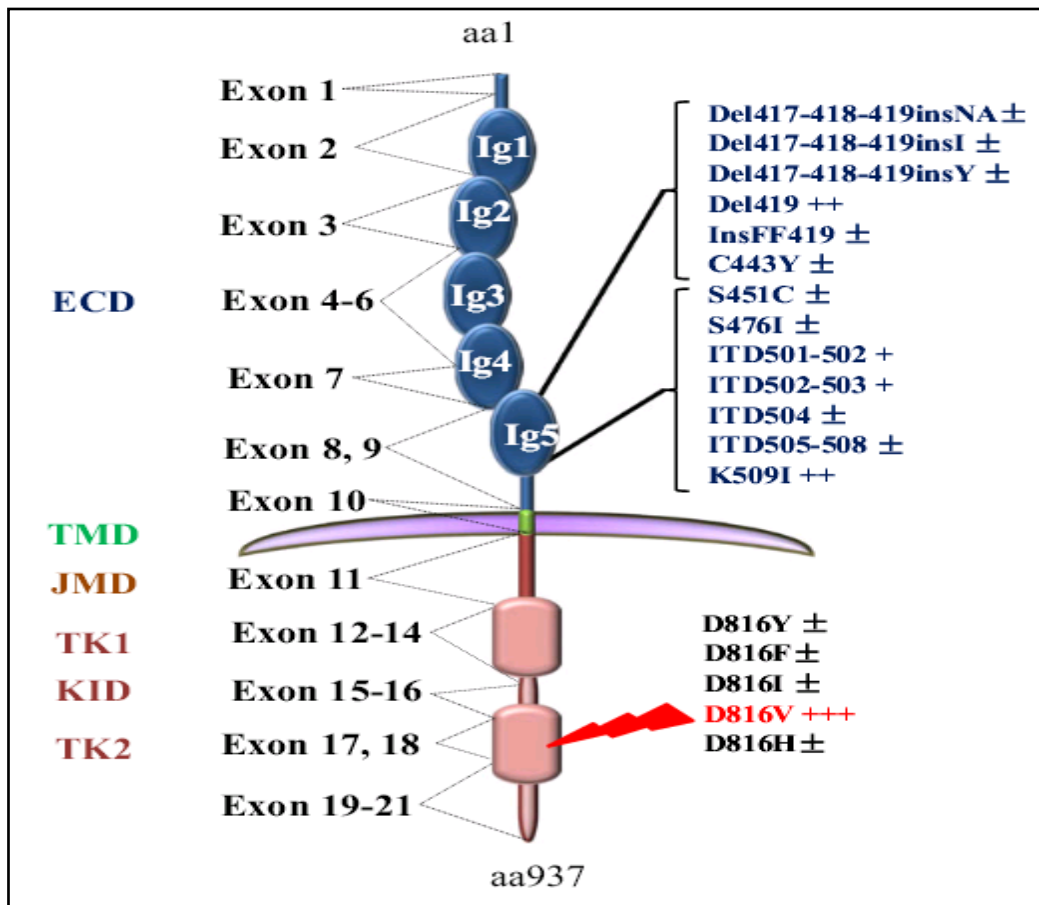


Figure 19: The different *KIT* mutations found in mastocytosis. In adults, the structure of *KIT* is found WT in <20% of all patients analyzed, whereas in children, *KIT* is found WT in around 25% of the patients analyzed. In adults, depending of the category of mastocytosis, the *KIT* D81V mutant (in red) is found in at least 80% of all patients, as mentioned in the text. Other *KIT* mutants in position 816 less frequently retrieved in mastocytosis are depicted here in black. In children, the ECD mutants (in blue) are found in nearly 40% of patients, whereas the *KIT* D816V mutant (in red) located in the phosphotransferase domain (PTD) is found in around 30% of the affected children. aa, amino acid; Del, deletion; Ins, insertion; ITD, internal tandem duplication; TK, tyrosine kinase. ±, mutation found in < 1% of the adult or pediatric patients; +, mutations found in 1–5% of pediatric patients; ++, mutation found in 5–20% of pediatric patients; +++, mutation found in >80% of all adult patients and in around 30% of pediatric patients (Figure from Arock *et al.* 2015).

Besides the “canonical” *KIT* D816V mutant, other rarest *KIT* mutations have been identified in a few patients and may also play a role in the etiology of mastocytosis. These mutations include V560G within the JMD of *KIT*; D816Y, D816F, and D816H in the PTD; the E839K dominant inactivating mutation in several cases of pediatric mastocytosis; and the rare germline mutation F522C in the TMD, which may be associated with WDSM. Exceedingly rare *KIT* mutations that are reported to be present in less than 1% of patients with SM include R815K, insV815_I816, I817V, D820G (all in the A-loop), del419 and K509I (in the ECD), and A533D and V559I in the JMD (Orafo *et al.* 2007).

Most cases of *KIT* mutations in the ECD were found in sporadic case in children (see above). In addition, these types of mutation have been described in familial mastocytosis. Regarding familial cases, they were reported in pediatric CM who present without *KIT* mutations or with uncommon mutations such as K509I, N835I, N835K, A533D, M835K, S841I, K839E or deletion of amino acids 419 or 559-560 (Longely *et al.* 1999; Zhang *et al.* 2006; Hoffmann *et al.* 2008; Wasag *et al.* 2011). Most of these mutations are of germline nature, activating and respond to imatinib treatment at least *in vitro* (Yang *et al.* 2010). Figure 19 shows the major *KIT* mutations found in patients with different categories of mastocytosis (Arock *et al.* 2015).

However, the situation in MCL contrasts to the one encountered in ISM. Indeed, *KIT* D816V is detected in only 46% of patients with MCL, the other patients having *KIT* WT and other *KIT* mutations in exon 9, exon 11 or exon 13 were found (Mital *et al.* 2011; Georjin-Lavialle *et al.* 2013). Only one case of MCL with *KIT* mutant in the ECD has been reported and found sensitive to imatinib (Mital *et al.* 2011). Besides, MCS patients show either no *KIT* mutation or non-816 *KIT* mutation such as *KIT* N822K or *KIT* Δ419 (Georjin-Lavialle *et al.* 2013).

2.5. Treatment of mastocytosis

Since mastocytosis is a heterogeneous disease presenting with a wide variety of clinical manifestations as mentioned above, its treatment is variable and based on the severity of clinical symptoms, patient complaints, and on prognosis (Valent *et al.* 2010; Arock *et al.* 2015; Pardanani 2015). Despite growing scientific knowledge, no curative therapy for mastocytosis exists (Escribano *et al.* 2002;

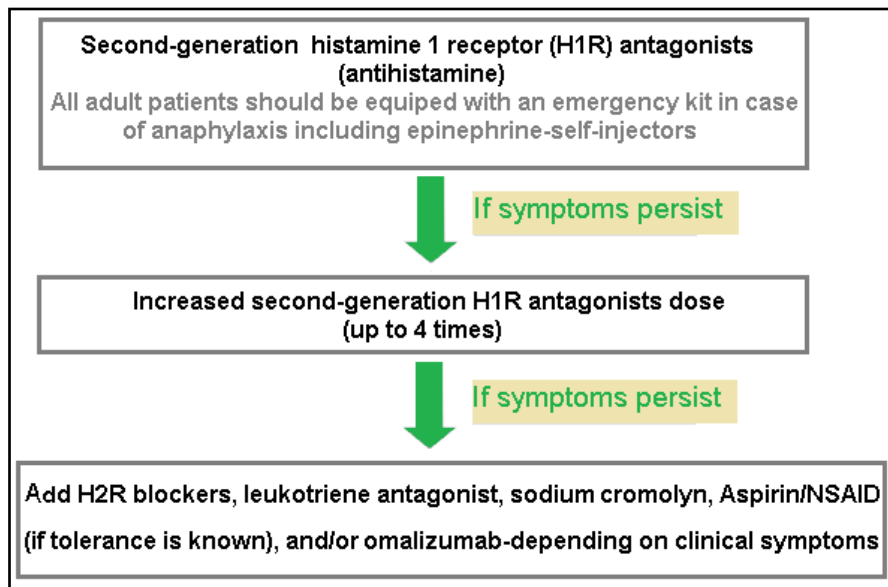


Figure 20: Proposed therapeutic algorithm for antimediation-treatment in mastocytosis (Figure from Siebenhaar & Akin 2014).

Brockow 2004), and the most important overall aim of treatment is the control of symptoms. Treatment recommendations for SM are based mostly on expert opinion rather than evidence obtained from controlled clinical trials and the condition is primarily managed by a hematologist (Wilson *et al.* 2006; Siebenhaar *et al.* 2014). Of note, in patients with SM-AHNMD, the SM component is treated as if no AHNMD was diagnosed, and AHNMD is treated as if no coexisting SM was found (Valent *et al.* 2010).

2.5.1. Symptomatic treatment of mastocytosis

In a substantial number of patients with CM or SM, diagnosed as CM_{SY} or SM_{SY}, mediator-related symptoms are recorded and cytoreductive therapy is not required. In these cases, the symptomatic treatment is required to control the mediator-related symptoms and is based on agents blocking the effects of MC-derived mediators, such as histamine receptor (H1R and H2R) antagonists, MC-stabilizing agents, antileukotrienes, glucocorticosteroids (GCs), and on bisphosphonates (Arock *et al.* 2015). Figure 20 depicts the proposed algorithm for antimediator-treatment in mastocytosis (Siebenhaar *et al.* 2014).

GI symptoms and ulcer can be treated by H2R antagonists including ranitidine and famotidine, proton pump inhibitors, antacids and oral sodium cromolyn, which has been shown to be effective in the control of diarrhea and abdominal cramping in many patients (Escribano *et al.* 2006).

Systemic administered corticosteroids can be effective in patients with frequent anaphylactic reactions or severe systemic conditions like ascites, diarrhea or malabsorption (Metcalf 1991).

In case of severe osteopenia, or frank osteoporosis, therapy with a bisphosphonate is recommended (Laroche *et al.* 2007; Arock & Valent 2010; Laroche *et al.* 2011). Prophylactic bisphosphonates are also recommended for SM patients who receive long-term GCs or have other (additional) risk factors (Arock & Valent 2010). Bisphosphonate also resolves pain in many SM patients suffering from unexplained pain accompanied by osteopenia (Lim *et al.* 2005).

In general, antimediator-type drugs are applied in a step-wise fashion and on the basis of the organ(s) affected (Valent *et al.* 2007). It is standard to instruct all patients to avoid agents, drugs, situations and

other symptom-trigger(s) for MC degranulation, such as NSAIDs, opioids, muscle relaxants, iodinated contrast agents, excessive heat or cold, and stress. Patients at risk for severe hypotension and vascular collapse should carry two or more epinephrine-self-injectors (adrenaline auto-injectors) and are taught to use them under prescribed conditions. In patients with IgE-dependent anaphylaxis, specific immunotherapy should be considered with recognition of potential risks (Valent *et al.* 2007). Recent reports have promised potential beneficial effects using omalizumab, a humanized anti-IgE antibody, in the treatment of mastocytosis (Siebenhaar *et al.* 2007; Douglass *et al.* 2010; Matito *et al.* 2013). In the absence of specific IgE, however, immunotherapy is not recommended. Also, cytoreductive agents are not recommended for treatment of mediator-related events (Valent *et al.* 2007; Pardanani 2015).

Oral psoralen plus UV-A (PUVA) therapy results in general and cosmetic benefits in the treatment of CM (Escribano *et al.* 2002; Worobec & Metcalfe 2002; Kinsler *et al.* 2005), however adverse effects are possible, such as skin cancer if more than 200 treatments are required. The manifestations of the disease usually recur several months after discontinuation of PUVA, but recurrences respond as well as the original lesions. Currently, PUVA therapy is reserved for severe, unresponsive cases in adults. Moreover, medium-dose UV-A1 therapy has been shown to be as effective as high-dose UV-A1 in reducing symptoms and the number of MCs in affected skin (Gobello *et al.* 2003). Also, treatment of cutaneous MC lesions with the 585-nm flashlamp-pumped dye laser and the 532-nm Nd:YAG laser has been reported to provide cosmetic improvement (Ellis 1996; Resh *et al.* 2005).

2.5.2. Non specific cytoreductive therapy

In patients with ASM, MCL and MCS, cytoreductive therapy is usually required since C findings are detected. These patients may benefit from non-targeted cytoreductive drugs, such as IFN- α (Kluin-Nelemans *et al.* 1992; Hauswirth *et al.* 2004; Tefferi *et al.* 2008; Pardanani 2015), 2-Chlorodeoxyadenosine (cladribine or 2-CdA) (Tefferi, Li *et al.* 2001; Kluin-Nelemans *et al.* 2003; Tefferi, & Pardanani 2004; Böhm *et al.* 2010; Arock & Valent 2010), or hydroxyurea (HU) (Valent *et al.* 2005).

2.5.2.1. Interferon (IFN)- α

Interferon (IFN)- α (IFN-alpha-2b (IFN- α 2b) in most cases) has formerly been considered first-line cytoreductive therapy in patients with ASM. It has been shown to improve symptoms of MC degranulation, UP and skin findings, symptomatic organomegaly and ascites, cytopenias, and osteoporosis, as well as to decrease BM MC infiltrations (Kluin-Nelemans *et al.* 1992; Worobec *et al.* 1996; Lehmann *et al.* 1996; Lippert & Henz 1996; Takasaki *et al.* 1998; Lehmann & Lämmle 1999; Hauswirth *et al.* 2004; Butterfield *et al.* 2005; Tefferi *et al.* 2008). However, IFN- α treatment is not uniformly effective, and the frequency of major response (MR) such as complete resolution of one or more C finding) is low (Valent *et al.* 2010; Pardanini 2015). Also, the optimal dose and duration of IFN- α treatment for patients with SM remain unclear. In addition, a significant proportion of patients will relapse within a short period of IFN- α treatment being discontinued, illustrating the cytostatic rather than cytolytic effects of the drug (Simon *et al.* 2004). Moreover, IFN- α treatment is frequently complicated by toxicities and adverse events including flu-like symptoms, bone pain, fever, cytopenias, depression, and hypothyroidism (Lippert & Henz 1996; Casassus *et al.* 2002; Lim *et al.* 2009; Arock *et al.* 2015). Nevertheless, concurrent administration of GCs may improve its efficacy (up to 40% MR rate) and tolerability (Delaporte *et al.* 1995; Hauswirth *et al.* 2004; Pardanini 2015).

2.5.2.2. 2-Chlorodeoxyadenosine (cladribine or 2-CdA)

Cladribine (2-CdA) has been shown to exert *in vivo* and *in vitro* inhibitory effects on the growth of neoplastic MCs (Böhm *et al.* 2010) and has been used in advanced SM subtypes including MCL (Penack *et al.* 2005), and as first-line treatment in SM patients with severe mediator-related symptoms refractory or intolerant to IFN- α (Tefferi *et al.* 2001; Kluin-Nelemans *et al.* 2003; Pardanani *et al.* 2004; Barete *et al.* 2015). Besides, 2-CdA has been combined with other chemotherapy prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Ustun *et al.* 2014).

Although 2-CdA has been reported to produce a long-lasting response with a sustained decrease in serum tryptase levels in two patients with progressive SSM as well as to be well tolerated with duration of remission in responding patients ranging from 2 months to 4 years (Böhm *et al.* 2010;

Pardanani *et al.* 2004), potential toxicities of this drug include myelosuppression and lymphopenia with increased risk of opportunistic infections (Hermine *et al.* 2010). In addition, in a study published by Kluin-Nelemans *et al.* (Kluin-Nelemans *et al.* 2003), none of the patients treated with 2-CdA achieved a complete remission (CR), indicating that 2-CdA is a non curative treatment option for severe SM. Moreover, 2-CdA was found ineffective in patients with progressive ASM or MCL (Böhm *et al.* 2010) as well as in SM-AHNMD (Hermine *et al.* 2010).

2.5.2.3. Hydroxyurea (HU)

Hydroxyurea (HU) is recommended as a reliable palliative drug in patients who are neither eligible for chemotherapy nor for HSCT (Valent *et al.* 2005). The HU has myelosuppressive activity, but does not exhibit any substantial anti-MC effect in SM-AHMD. It has been used as first-line therapy in patients with SM-AHNMD in a Mayo Clinic study. Median duration of response was 31.5 months (range 5–50 months) and the major toxicity was myelosuppression (Lim *et al.* 2009).

2.5.3. Novel approaches in the treatment of mastocytosis: targeted therapies

During the last few years, a number of different targeted drugs targeting tyrosine kinase (TK) activity have been used in patients with advanced MC disorders and several inhibitors against the mutated form of KIT have been developed.

2.5.3.1. Imatinib

The TK inhibitor imatinib (STI571) (400 mg per day) is approved by the Food and Drug Administration (FDA) for use in ASM patients without *KIT* D816V mutation or with unknown *KIT* mutation status, but with organ dysfunction due to progressive infiltration of various organs by MCs (Bains & Hsieh 2010). However, the role of imatinib in the treatment of ISM and CM is less well established. Aqarwala *et al.* have demonstrated the usefulness of imatinib in the treatment of *KIT816*-unmutated ISM with extensive cutaneous involvement (Aqarwala *et al.* 2013). Moreover, patients with WDSM respond to imatinib mesylate therapy (Alvarez-Twose *et al.* 2012). In fact, imatinib is an

effective inhibitor of the WT KIT TK as well as of the BCR-ABL and PDGFR TKs (Pardnani & Tefferi 2004; Hoffmann *et al.* 2008; Pardnani 2013). Imatinib has no effectiveness in mastocytosis patients with *KIT* D816V mutation (Frost *et al.* 2002; Akin *et al.* 2003; Heinrich *et al.* 2008; Lim *et al.* 2009) since this mutation leads to a steric alteration in the receptor preventing imatinib from binding to the ATP binding domain of KIT, as outlined above (Ma *et al.* 2002; Vendome *et al.* 2005). However, patients carrying uncommon *KIT* mutations in exons 9 or 10 such as F522C, K509I, del419 mutations, as well as the JMD mutations V560G and V559G, but not V559I respond to imatinib which has been found to reduce MC burden, to lower tryptase levels and to improve the clinical symptoms (Akin *et al.* 2004; Zhang *et al.* 2006; Nakagomi & Hirota 2007; Hoffmann *et al.* 2008; Ashman & Griffith 2013; de Melo Campos *et al.* 2014). Finally, the SM patients with *FIP1L1-PDGFR*A mutation responds well to imatinib mesylate therapy (Tefferi *et al.* 2008).

2.5.3.2. Masitinib

Masitinib (AB1010) is another KIT inhibitor that was first registered to treat canine MC tumors (Hahn *et al.* 2008). It has *in vitro* activity against KIT WT in human MCs greater than that of imatinib, and it also inhibits PDGFR and Lyn (Dubreuil *et al.* 2009). In contrast, masitinib demonstrates weak inhibition of ABL and c-Fms and does not block the *KIT* D816V mutant. However, the highly selective nature of masitinib suggests that it will exhibit a better safety profile than other TK inhibitors; indeed, masitinib-induced cardiotoxicity or genotoxicity has not been observed in animal studies (Dubreuil *et al.* 2009). Masitinib inhibits more strongly degranulation, cytokine production, and BM-derived MC migration than imatinib. Furthermore, it potently inhibits human and murine KIT with JMD activating mutations (Dubreuil *et al.* 2009) and is considered a promising treatment for indolent forms of mastocytosis with handicap (Paul *et al.* 2010). Georjin-Lavialle *et al.* have recently reported that masitinib is safe and effective for the treatment of a MCL patient having a new KIT mutation, dup(501-502) (Georjin-Lavialle *et al.* 2012). A phase III study of masitinib in patients with SSM, ISM, or CM is currently recruiting (NCT00814073).

2.5.3.3. Dasatinib (BMS354825)

Dasatinib is an orally available short-acting dual ABL/Src TK inhibitor (Lindauer & Hochhaus 2014) that is more potent than imatinib on a molar basis against the BCR-ABL kinase (Olivieri & Manzione 2007). Dasatinib is also highly active against PDGFRA and has shown efficacy *in vitro* against various *KIT* mutants including D816V (Schittenhelm *et al.* 2006; Shan *et al.* 2006; de Melo Campos *et al.* 2014). In addition, dasatinib exerts *in vitro* cytotoxic effects against primary neoplastic MCs derived from the BM of patients with SM (Gleixner *et al.* 2013). Furthermore, dasatinib may synergize with PKC412 and chemotherapy in this regard (Gleixner *et al.* 2007; Aichberger *et al.* 2008; Ustun *et al.* 2009). However, based upon data of phase II study of dasatinib in Philadelphia chromosome-negative acute and chronic myeloid diseases, including SM, dasatinib therapy may benefit to a selected group of SM patients, primarily by improving their symptoms, but it does not eliminate the disease in the patients with *KIT* D816V mutation (Verstovsek *et al.* 2008). Purtil *et al.* have reported that dasatinib exhibits modest activity in patients with SM *in vivo* (Purtill *et al.* 2008). In a patient with rapidly progressing ASM, neither dasatinib nor 2-CdA produce a long-lasting response *in vivo*, despite encouraging *in vitro* results.

In addition, dasatinib induces several adverse effects, particularly pleural effusions, in a substantial number of patients (Lindauer & Hochhaus 2014). Notably, responders in the phase II study of dasatinib were found to experience an initial exacerbation of MC mediator-release symptoms (MCMRS) and rash lasting several days before the benefit benefits of dasatinib therapy became evident (Verstovsek *et al.* 2008). Thus, dasatinib appears to date to have modest activity in *KIT*D816V+ SM (Pardanani 2015).

2.5.3.4. Midostaurin (PKC412)

Midostaurin (PKC412) is a potent multikinase inhibitor that demonstrates *in vitro* activity against KD *KIT* mutants (D816Y and D816V) (Growney *et al.* 2005; Gleixner *et al.* 2006). It is also active against PKC, fms related tyrosine kinase 3 (FLT3), PDGFRA, and vascular endothelial growth factor receptor 2 (VEGFR-2) (Fabbro *et al.* 2000).

Midostaurin abrogates the phosphorylation of KIT in HMC-1.1 and HMC-1.2 cells in a time-dependent and dose-dependent manner and suppresses the growth of primary neoplastic human MCs harboring either KIT D816V or WT KIT (Gleixner *et al.* 2013). In addition to its growth-inhibitory effects, midostaurin has been described to inhibit histamine secretion from activated normal and neoplastic MCs. Thus, it may be a beneficial drug in ASM suffering from mediator-related symptoms (Krauth *et al.* 2009). Of note, midostaurin can act synergistically with nilotinib and dasatinib to inhibit the growth of neoplastic MCs with the *KIT* D816V mutation (Gleixner *et al.* 2006; Gleixner *et al.* 2013).

Interestingly, midostaurin induced remission in a patient with MCL carrying the activating *KIT* D816V mutation (Gotlib *et al.* 2005) and several clinical trials are ongoing to determine the efficacy and tolerability of midostaurin in patients with SM (Knapper *et al.* 2011; Gotlib *et al.* 2014). Initial results from a phase II study of single-agent midostaurin showed promising activity in 26 patients with advanced SM, with a 69% response rate: 38% major response, 19% good partial response, and 12% minor partial response. Importantly, all patients who achieved a major response had the *KIT* D816V mutation, and the response was durable for a median of 1.5 year as well as Midostaurin was relatively well tolerated (Gotlib *et al.* 2010). A larger international phase II clinical trial in patients with ASM or MCL (National Clinical Trial NCT00782067) presented at the 2012 American Society of Hematology Annual Meeting, demonstrated that midostaurin was associated with durable responses in patients with advanced SM, particularly in MCL (Gotlib *et al.* 2012). In addition, another study has recently demonstrated that, despite the absence of complete remission, PKC412 gives survival advantage for patients with advanced SM over patients who did not receive the drug (Chandesris *et al.* 2014). However, the long-term benefit of PKC412 has not yet been determined. Therefore, development of novel effective drugs to treat ASM and MCL is still needed.

2.5.3.5. Nilotinib (AMN107)

Nilotinib is a TK inhibitor that also inhibits KIT, BCR-ABL, and PDGFR, (Manely *et al.* 2010). Low concentrations of nilotinib reduced the growth of and induced apoptosis in transformed murine Ba/F3

cells expressing the *Kit* D814V mutation, which corresponds to the human *KIT* D816V mutation (von Bubnoff *et al.* 2005). However, nilotinib was not sufficient to inhibit the growth of *KIT* D816V–positive BM MCs obtained from patients with ASM, but showed activity in cell lines expressing *KIT* WT and the very rare JM mutation (*KIT* D560G) (Verstovsek *et al.* 2006). In a phase 2 clinical study, an overall response rate (ORR) of 20% was seen in patients with SM (83% *KIT* D816V positive) treated with nilotinib and included two complete responses, one partial response, five incomplete responses, and four minor responses (Hochhaus *et al.* 2006).

2.5.3.6. Ponatinib (AP24534)

Ponatinib (AP24534) is a potent multitargeted kinase inhibitor that has been approved by the FDA for treatment of imatinib-resistant patients with CML, including those harboring the *BCR-ABL* T315I mutant (O’Har *et al.* 2009; Cortes *et al.* 2012). Ponatinib has inhibitory effects on a wide variety of kinase targets, including *KIT*, *FLT3*, fibroblast growth factor receptor 1 (*FGFR1*), and platelet-derived growth factor receptor α (*PDGFR* α) (O’Har *et al.* 2009; Gozgit *et al.* 2011; Gozgit *et al.* 2012; Lierman *et al.* 2012) and blocks several downstream signaling molecules, including *Lyn* (Gozgit *et al.* 2011; Lierman *et al.* 2012; Okabe *et al.* 2013).

It has been reported that ponatinib is active against BaF3 cells stably expressing *KIT* Y823D *in vitro* (Lierman *et al.* 2012), inhibits the growth and survival of *KIT* D816V positive neoplastic MCs and can produce synergistic growth-inhibitory effects when combined with PKC412 (Gleixner *et al.* 2013). Furthermore, ponatinib inhibits the growth of primary neoplastic MCs, and abrogates the phosphorylation of *KIT* harboring the point mutation V560G (but less effectively the D816V mutation) and the downstream signaling transduction (Gleixner *et al.* 2013; Jin *et al.* 2014). Interestingly, ponatinib was also found to inhibit the growth of xenografted HMC-1.2 cells in nude mice and significantly prolong the survival of mice with ASM or MCL (Jin *et al.* 2014).

However, whether ponatinib, alone or in combination, is able in clinical trials to suppress the *in vivo* growth of neoplastic MCs in patients with advanced SM and whether potential benefits could exceed the risk of its adverse effects such as thrombotic vascular events remain to be examined.

2.5.3.7. Novel TKIs

Tandutinib (MLN518) was found to inhibit the proliferation of KIT D816V positive Ba/F3 cells and P815 cells (Corbin *et al.* 2004). Semaxinib (SU5416) induces apoptosis in *KIT D816V* positive erythroleukemic cells and inhibits KIT autophosphorylation as well as activation of AKT, ERK1/2 and Stat3 downstream signaling pathways (Kosmider *et al.* 2007). EXEL-0862 was also found to induce apoptosis *in vitro* and *ex vivo* and to decrease phosphorylation of STAT3 and STAT5 in HMC-1.2 cell line (Pan *et al.* 2007).

The dual Bcr-Abl and Lyn-kinase inhibitor bafetinib (INNO-406) was found to inhibit proliferation and block KIT phosphorylation in HMC-1.1 cells, but not in HMC-1.2 cells or primary neoplastic cells in patients with KIT D816V-positive SM but it block LYN phosphorylation in both cell lines (Peter *et al.* 2010).

Besides, the adenosine triphosphate (ATP)-based kinase inhibitors (AP23464, AP23848) showed *in vitro* and *in vivo* activity against activation-loop mutants of KIT (Corbin *et al.* 2005).

In addition, the TK inhibitor APcK110 induces apoptosis and inhibits proliferation of HMC-1.2 cells (Faderl *et al.* 2009). Moreover, both MLN518 and PD180970 compounds inhibit the growth of HMC-1.1 cell line. MLN518 additionally targets active site mutant cell lines, inhibits the proliferation of *KIT D816V* positive Ba/F3 cells and P815 cells, abolishes KIT and STAT3 phosphorylation and induces apoptosis. This drug was found well tolerated in phase 1 clinical trials in AML (Corbin *et al.* 2004). The small molecule TK inhibitor, bosutinib, is currently approved for CML treatment (Ai & Tiu 2014). It deactivates Lyn and Btk without blocking KIT activity and can inhibit the growth of neoplastic MCs *in vitro* (Gleixner *et al.* 2009; Gleixner *et al.* 2011). Moreover, bosutinib cooperates with PKC412 to block the growth of *KIT*-mutated neoplastic MCs (Gleixner *et al.* 2011).

Interestingly, the thiazole amine class TK inhibitor 126332 has been described to potently inhibit the proliferation of *KIT D816V*-expressing cells as well as the phosphorylation of KIT and of its downstream signaling molecules STAT3 and STAT5. Compound 126332 induced apoptosis and downregulated levels of Mcl-1 and survivin. Furthermore, this molecule exhibited also *in vivo* antineoplastic activity against cells harboring the *KIT D816V* mutation (Jin *et al.* 2014).

However, all TKIs cited above still need to be evaluated in clinical trials to explore their real antineoplastic potential in ASM and MCL.

2.5.4. Allogeneic hematopoietic stem cell transplantation (allo-HSCT)

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is considered as a viable and potentially curative therapeutic option for aggressive hematologic malignancies (Holtick *et al.* 2015). In fact, Allo-HSCT remains the only potentially curative treatment option for patients with advanced SM. The effect of allo-HSCT in patients with advanced SM (SM-AHNMD, n=38; MCL, n=12; ASM, n=7) has been recently reported in a global multi-center study (Ustun *et al.* 2014). The median age of patients was 46 years. Most patients received a graft from HLA (HumanLeukocyte Antigens)-identical siblings (n=34) or unrelated donors (URD) (n=17). Myeloablative (n=36) conditioning (MAC) was more commonly used. Responses (defined *per* decrease in serum tryptase level and in BM MC percentage) were observed in 40 patients (70%), including 16 patients (28%) with CR. Overall survival (OS) and SM progression-free survival (PFS) at 3 years were 57% and 51% for all patients. OS and PFS were significantly affected by the type of advanced SM: 74% and 63% for SM-AHNMD, 43% and 43% for ASM, and 17% and 17% for MCL. Risk factors for poor survival included MCL (the most important factor), lower Karnofsky performance status, and patients receiving reduced-intensity conditioning regimens. Acute graft-versus-host disease (GVHD) grades II-IV and chronic extensive GVHD occurred in 40% and 23% of patients, respectively. Treatment-related mortality (TRM) at 1 year was 20% (MCL patients had the highest rate: 33%). Thus, allo-HSCT should be considered in all patients with drug-resistant advanced SM who are young and fit and have a suitable donor. Although the data presented are very encouraging, future prospective studies are required to confirm the value and efficacy of this treatment approach in such categories of SM.

2.5.5. Potential alternative therapeutic targets

Given the fact that the neoplastic MCs can rely on KIT-independent signaling pathways for survival, and that these pathways possibly contribute to disease progression (Gleixner *et al.* 2007), many patients treated with TKI relapse and thus current studies focus on drug combinations. Combinations of targeted drugs may represent an interesting pharmacologic approach for the treatment of aggressive SM or MCL. However, most potent TKIs and drug combinations should be tested in clinical trials. Moreover, another interesting approach may be to attack neoplastic MC and their progenitor cells (SM stem cells) by targeting antibodies directed against various neoplastic MC-restricted surface antigens which constitute a viable option for the treatment of SM and warrant further investigations.

Indeed, many alternative targets of interest are overexpressed either on or in the neoplastic MCs cells compared to normal MCs (Valent *et al.* 2010). Some of these targets are thought to contribute to the mechanism(s) involved in signaling pathways critical for the transforming abilities of mutant KIT, such as the PI3-kinase-AKT pathway. Besides, signal transduction molecules that play a pivotal role in the growth, survival, and transformation of neoplastic MCs, such as FES, the transcription factor STAT5, or the mTOR complex represent promising and novel targets. The potential functional and clinical role of some of these targets has been recently demonstrated in *in vitro* and *ex vivo* studies, when used alone or in combination with KIT inhibitors. In addition, for certain targets, specific drugs are already available that might be tested in the future in clinical trials if all the preclinical studies appear convincing.

2.5.5.1. Targets related to signaling or apoptosis

Since AKT has been reported to be involved in KIT-dependent differentiation and growth of neoplastic MCs harboring oncogenic KIT mutants, and to be phosphorylated in the HMC-1.2 cell line as well as in neoplastic MCs from patients suffering from KIT D816V+ SM, its activation is considered to play potentially a critical role in the pathogenesis of mastocytosis (Harir *et al.* 2008; Grimwarde *et al.* 2009; Yang *et al.* 2010). Of note, AKT inhibitors decrease the proliferation of *KIT* D816V+human MCs *in vitro* (Saleh, wedeh *et al.* 2014).

The heat shock protein 90 (hsp90) inhibitor-17-demethoxygeldanamycin (17-AAG) binds to hsp90 and leads to the destabilization of various hsp90-dependent kinases important in oncogenesis. Treatment of both HMC-1.1 cell line and with HMC-1.2 cell line with 17-AAG downregulates both the level and activity of KIT and downstream signaling molecules AKT and STAT3, indicating that 17-AAG may be effective in the treatment of SM, and MCL (Fumo *et al.* 2004).

It is well known today that FES TK is phosphorylated on tyrosine residues in *KIT* D816V+ cells and reduction of FES expression using RNA interference (RNAi) decreases also the proliferation of these cells (Voisset *et al.* 2007). However, to date there is no specific FES inhibitor.

As mentioned above, rapamycin inhibits mTOR and thus the survival of *KIT* D816V+ MCs (Gabillot-Carré *et al.* 2006). Inhibition of mTORC2 *via* a targeted approach may reduce the proliferation in MC disorders without affecting the normal differentiated MCs and therefore might be a promising therapeutic alternative in the future (Smrz *et al.* 2011).

Regarding STAT5, it is well admitted that its expression plays a critical role of in MC development and survival (Shelburne *et al.* 2003; Chaix *et al.* 2011). Interestingly, it has been reported that phosphorylated STAT5 (p-STAT5) is found in the cytoplasm of neoplastic MCs from patients with SM (Harir *et al.* 2007; Baumgartner *et al.* 2009) and that knockdown of STAT5, AKT, or both resulted in cell growth inhibition (Harir *et al.* 2008). Moreover, it has been reported that *KIT* D816V promotes the direct activation of STAT5 and that p-STAT5 contributes to growth of neoplastic MCs as shown using knockdown experiments (Harir *et al.* 2008). Recently, STAT5 has been reviewed to be one major cellular effector in mastocytosis by controlling the mutant *KIT*-mediated aberrant growth signaling. However, the pharmacological inhibition of STAT5 remains challenging, and new STAT5 inhibitors active at pharmacological doses in both indolent and aggressive forms of SM are still required (Bibi *et al.* 2014).

The BH3-only (BCL-2 homology) proteins, a group of proapoptotic factors, have been described to modulate MC survival *in vitro* and *in vivo* (Hartmann *et al.* 2003; Ekoff *et al.* 2007; Aichberger *et al.* 2009; Karlberg *et al.* 2010; Peter *et al.* 2014). Targeting of BCL-2 family members by drugs promoting BIM (re)-expression, or by BH3-mimetics such as obatoclax, may thus be an attractive

therapy concept in SM. In deed, the proapoptotic BCL-2 family member BIM has been identified as a tumor suppressor in neoplastic MCs as it is down-regulated in neoplastic MCs by SCF stimulation and by the oncogenic KIT D816V TK. Besides, midostaurin and the proteasome inhibitor bortezomib up-regulate BIM in HMC-1.1 and HMC-1.2 cells (Aichberger *et al.* 2009).

Recently, neoplastic MC in ASM and MCL were found to express antiapoptotic MCL-1, BCL-2, and BCL-xL and the pan-BCL-2 family blocker obatoclax (GX015-070) was found to inhibit the proliferation of primary neoplastic MCs and of HMC-1 cell lines. Obatoclax was also found to induce apoptosis and to increase the expression of PUMA, NOXA, and BIM mRNA. Moreover, obatoclax synergizes with several other antineoplastic drugs, including dasatinib, midostaurin, and bortezomib, in producing apoptosis and/or growth arrest in neoplastic MC (Peter *et al.* 2014).

The autophosphorylated KIT receptor induces NF- κ B activation and results in the up-regulation of cyclin D3 expression and cell cycle progression. Therefore, compounds that interfere with NF- κ B signaling may present a therapeutic potential in SM. The NF- κ B inhibitor IMD-0354 has been documented to restrain *in vitro* factor-independent proliferation of neoplastic MCs with *KIT* mutations but not of normal MCs (Tanaka *et al.* 2005).

Moreover, human MCs have recently been reported to express receptors that could allow for the induction of apoptosis and inhibition of activation, like the Tumor Necrosis Factor Related Apoptosis Inducing Ligand receptor (TRAIL) and the inhibitory receptors CD300a and Siglec-8 (Karra *et al.* 2009; Karra & Levi-Schaffer 2011; Migalovich-Sheikhet *et al.* 2012). So far, TRAIL receptor is the only death receptor known to be expressed on human MCs and, therefore, constitutes an interesting therapeutic target. Besides, the recently described inhibitory receptors, CD300a and Siglec-8, have potential downregulatory properties on MC activation and survival *in vivo* (Berent-Maoz *et al.* 2010). A selective targeting of these receptors could lead to a potential innovative treatment strategy in mastocytosis (Karra *et al.* 2009).

Furthermore, pimecrolimus and other calcineurin inhibitors or dimethylfumarate, have been reported to induce apoptosis in MCs and in disease models of mastocytosis may be another potential therapy for mastocytosis and other MC-associated diseases (Ma *et al.* 2010; Ma & Jiao 2011; Förster *et al.*

2013).

Other substances have been tested for their efficacy in pilot clinical trials, such as the raft modulator miltefosine. Miltefosine has been shown to reduce the release of histamine, TNF- α , and PGD₂ from MCs upon IgE-mediated and non-IgE-mediated activation. In addition, topical administration of miltefosine induced a reduction of histamine-induced wheal-and-flare-type skin reactions *in vivo*. In a randomized, placebo-controlled, double-blind clinical trial, topical miltefosine has been shown to inhibit MC activation in patients with CM (Weller *et al.* 2010; Hartmann *et al.* 2010; Magerl *et al.* 2012; Maurer *et al.* 2013).

2.5.5.2. Antigen targeting

The cell surface antigens expressed on neoplastic MCs and/or their progenitor cells such as CD13 (aminopeptidase N), CD25, CD30, CD33, CD44, CD52, CD87, and CD117 have been considered as potential targets of antibody-based therapy in advanced MC disorders (Sotlar *et al.* 2004; Valent *et al.* 2010; Valent *et al.* 2011; Arock *et al.* 2015). In fact, CD30, CD33, and CD52 are considered the most promising and/or relevant antigens in the treatment of mastocytosis (Arock *et al.* 2015). Neoplastic MCs in patients with ASM and MCL express CD30 and CD52 at higher levels than those of MCs from patients with ISM (Valent *et al.* 2011; Sotlar *et al.* 2011; Hoermann *et al.* 2014). In physiologic conditions, CD30 is infrequently expressed and thus is considered a specific and useful target in advanced SM (Van Anrooij *et al.* 2014). Recently, a single-arm open-label trial clinical trial has been initiated (and is still ongoing) to study the potential effects of Brentuximab Vedotin (a CD30-targeted antibody conjugated with the antimetabolic agent auristatin E) in CD30-positive SM with or without an AHNMD (National Clinical Trial NCT01807598).

Because of its expression on the putative neoplastic stem cells in SM, CD33 is considered as an attractive target in mastocytosis (Florian *et al.* 2006). Interestingly, neoplastic MCs and their progenitors respond *in vitro* to Mylotarg, a drug composed by a humanized CD33 antibody (gemtuzumab) and the cytostatic agent calicheamicin (ozogamicin) (Krauth *et al.* 2007). However, whether patients with advanced SM may benefit of Mylotarg is still unknown.

The cell surface antigen CD52 (CAMPATH-1) has been recently identified as a molecular target expressed abundantly on the surface of primary neoplastic MCs in patients with advanced SM (Hoermann *et al.* 2014). The CD52-targeting antibody alemtuzumab has been found to induce cell death in CD52+ primary neoplastic MCs obtained from patients with SM as well as an immature human MC line (MCPV-1). Moreover, alemtuzumab improves significantly the survival of NSG xenotransplanted with MCPV-1 cells (Hoermann *et al.* 2014).

To conclude, current therapy for patients with SM refractory to interferon- α or cladribine is inadequate and it is recommended to involve such patients in experimental treatment protocols (Tefferi *et al.* 2008). Patients with very poor prognostic ASM, MCL or SM-AHNMD (because of the AHNMD) should be considered for enrollment in prospective clinical trials. Therefore, there is still a need to identify novel druggable targets in neoplastic MCs and their progenitors in order to optimize therapy and to prolong survival of these patients. The promising novel targets should be thereafter evaluated alone or in combination with TKIs or other chemotherapy in patients with advanced SM.

3. Bromodomain containing protein 4 (BRD4)

3.1. Bromodomains (BRDs): readers of acetyl lysine residues

Epigenetics has been defined as heritable changes in the pattern of post-translational modifications (PTMs, also referred to as the epigenetic codes), which lead to the development of new phenotypes that are not encoded in the DNA sequences (Holliday 1987). Readers of PTMs are structurally diverse proteins that contain one or more effector modules that recognize and read covalent modifications of proteins and DNA (Filippakopoulos & Knapp 2014). Acetylation of lysine residues, the first PTMS of histone described in 1964 (Allfrey *et al.* 1964) is a reversible and widespread PTM, and extensively relevant to modulation of cellular processes, including protein conformation and interaction (Kouzarides 2000; Choudhary *et al.* 2014). Moreover, lysine acetylation is abundant in large macromolecular complexes that function in chromatin remodeling, DNA damage, and cell cycle control (Choudhary *et al.* 2009) and particularly in histones.

Of note, three types of proteins have been identified to regulate lysine acetylation including bromodomain (BRD) proteins (Barneda-Zahonero & Parra 2012; Filippakopoulos & Knapp 2014), histone acetyltransferases (HATs), histone deacetylases (HDACs) (Shahbazian & Grunstein 2007) and sirtuins (SIRTs) (Dhalluin *et al.* 1999; Anand *et al.* 2013; Seto & Yoshida 2014; Chen *et al.* 2014). Indeed, BRD proteins act as readers of lysine acetylation state as they bind acetylated lysine residues (K_{Ac}). The HATs act as writers as they perform the enzymatic transfer of an acetyl group from the acetylCoA to the ϵ -amino group of lysine side chains of histone protein tails and display target selectivity (Shahbazian & Grunstein 2007), whereas HDACs and SIRTs remove acetyl groups and act as erasers (Anand *et al.* 2013; Fu *et al.* 2015).

Lysine acetylation leads to neutralization of the basic charge of the lysine residues and changes the electrostatic interaction between histones or histones and DNA and hence increases DNA accessibility to the binding of transcriptional factors, and is thus associated with activated states of chromatin by changing the nucleosome positioning and chromatin architecture (Turner 2000). More importantly, lysine acetylation provides an instructive pattern for the recruitment to chromatin of factors involved not only in transcriptional regulation, but also in DNA repair and replication (Kouzarides 2007; Shahbazian & Grunstein 2007).

The recognition of ϵ -*N*-acetylation of lysine residues is primarily initiated by bromodomains (BRDs). BRDs are small (of about 110 amino acids in length) evolutionarily and structurally conserved protein interaction modules that were originally identified in the early 1990s by sequence alignment as a common motif in the *brahma* gene from *Drosophila melanogaster* (Tamkun *et al.* 1992), female-sterile homeotic (*fsh*) and four other potential transcriptional regulators (Haynes *et al.* 1992). Additionally, the recognition of histone acetylation code by BRDs is selective, involved in transcription, and potentially conveys transcriptional memory across cell divisions (Kanno *et al.* 2004). In fact, the binding interaction of BRDs with acetylated histones leads to the recruitment of additional proteins to specific sites on chromatin and, as the coassociated proteins may have enzymatic activity; this can lead to additional chromatin modification. For example, where a BRD module is part of a HAT, histone acetylation can serve as a regulator of the HAT activity (Winston & Allis 1999).

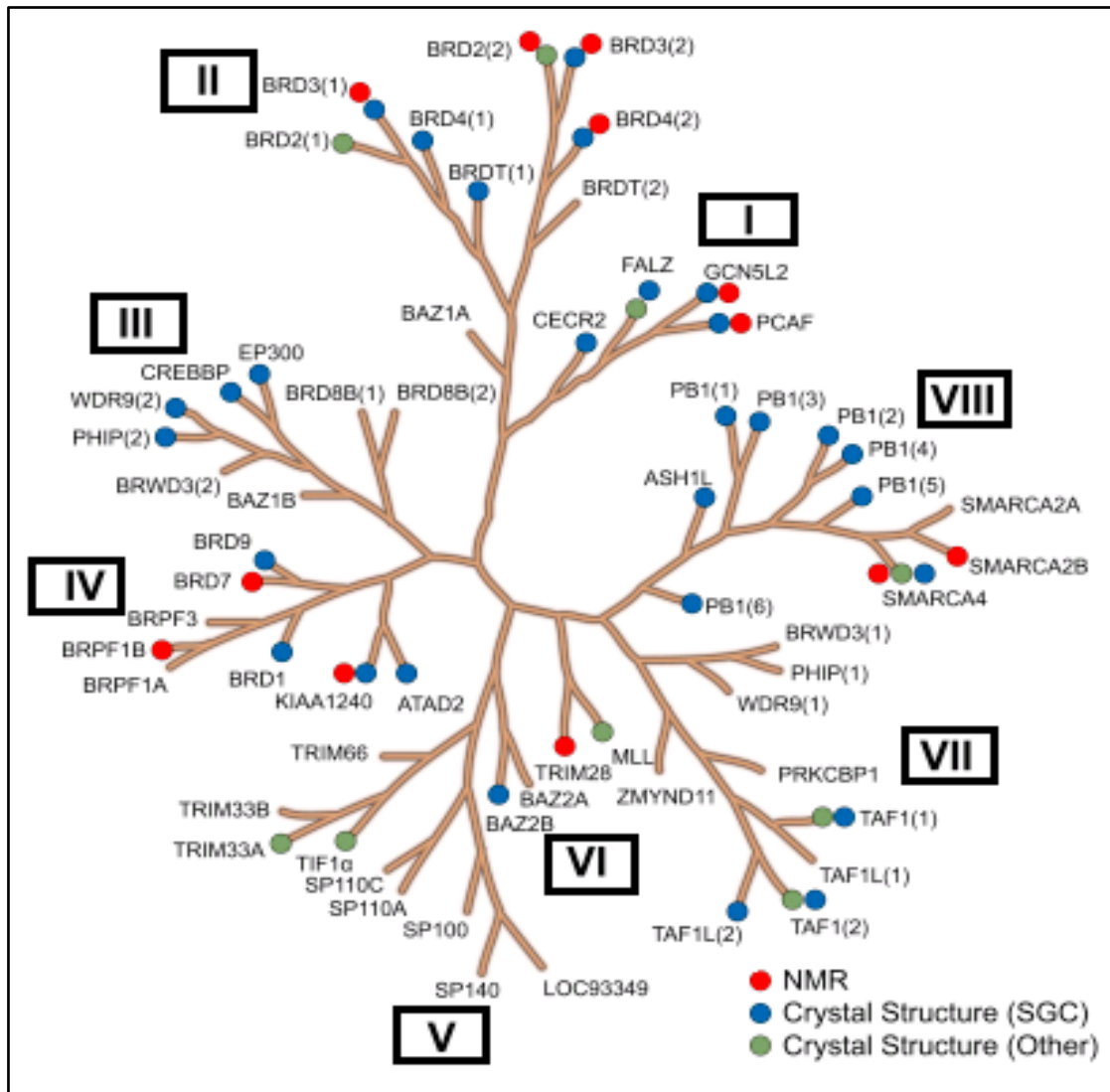


Figure 21: Phylogenetic tree of the human BRD family based on sequence alignments of predicted BRDs. The different families are named by Roman numbers (I–VIII). Structures determined in this study, by NMR, or by other groups are indicated by blue, red, and green dots, respectively. For targets with multiple BRDs, the domains have been numbered starting from the N-terminus and the number is shown in parentheses (Figure adapted from Filippakopoulos *et al.* 2012 and from Prinjha *et al.* 2012).

Further, cooperative binding to other chromatin associated proteins helps achieving a high level of targeting specificity (Garnier *et al.* 2014).

The BRDs are most commonly found as single copies, frequently in combination with other epigenetic reader domains such as PHD (plant homeodomain), PWWP (named for conserved pro-trp-trp-pro motif) and Tudor (Chung 2012). In fact, the PHD finger, which is a zinc-finger-like motif present in nuclear proteins, is considered the most frequently associated domain with BRDs, whilst the second most common association is another BRD (Garnier *et al.* 2014).

Furthermore, the human genome encodes 61 BRDs, which are present in 46 different nuclear and cytoplasmic proteins (Filippakopoulos & Knapp 2014; Shi & Vakoc 2014; Gallenkamp *et al.* 2014), where differences in the amino acid residues around the acetyl lysine binding site impact ligand specificity (Filippakopoulos *et al.* 2012). Proteins containing this module are often involved in transcriptional regulation and chromatin remodeling (Garnier *et al.* 2014). Moreover, structure-based alignments have clustered human BRD families into eight distinct families by the derived phylogram analysis in which the BRDs and extra-terminal domain (BET) subfamily of proteins discussed later belongs to the subfamily II as shown in Figure 21. Indeed, BRDs have been found in:

- histone acetyltransferases (HATs) and HAT-associated proteins (such as general control of amino acid synthesis protein 5-like 2 (GCN5L2; also known as KAT2A),
- P300/CBP-associated factor (PCAF; also known as KAT2B) and bromodomain-containing protein 9 (BRD9)) (Yang *et al.* 1996; Dhalluin *et al.* 1999),
- histone methyltransferases (such as ASH1L and mixed lineage leukemia protein (MLL)) (Gregory *et al.* 2007; Malik & Bhaumik 2010),
- helicases (such as SWI/SNF (switch mating type/sucrose non-fermenting)-related matrix-associated actin-dependent regulators of chromatin subfamily A (SMARCA5)) (Trotter *et al.* 2008),
- ATP-dependent chromatin remodelling complexes (such as bromodomain adjacent to zinc finger domain protein 1B (BAZ1B; also known as Williams syndrome transcription factor)) (Cavellán *et al.* 2006),
- transcriptional co-activators (such as tripartite motif-containing proteins (TRIMs) and TBP-

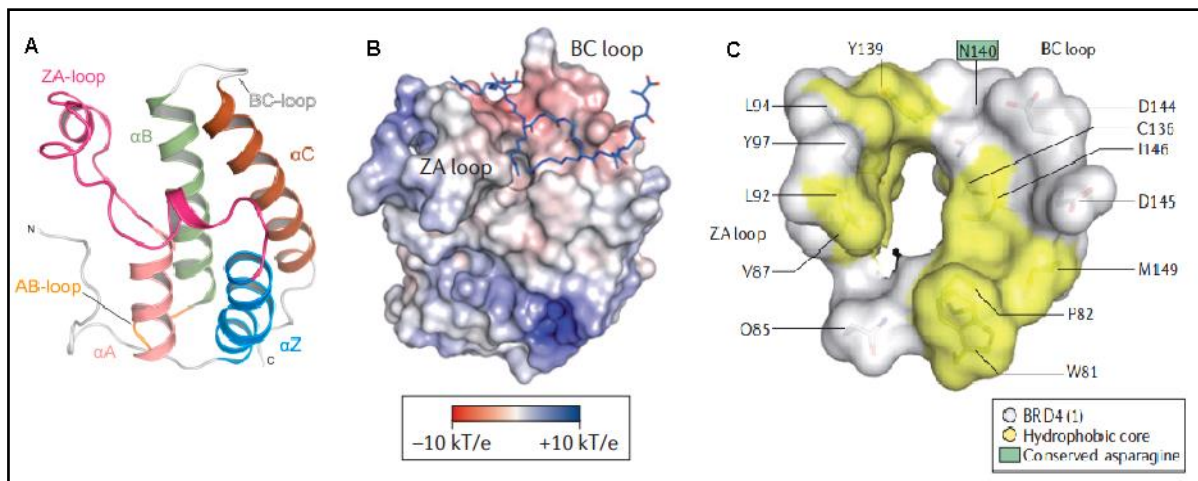


Figure 22: Structure of BRD module. A. The BRD is a highly conserved motif of 110 amino acids which is bundled into four anti-parallel α -helices that are linked by diverse loop regions (ZA and BC loops). B. Major structural elements are highlighted on the structure of the first bromodomain of bromodomain-containing protein 4 (BRD4(1)) in complex with a di-acetylated histone H4 peptide. The electrostatic potential of the module is also shown, from -10 to $+10$ kT/e, highlighting the charged nature of the surface lining the acetyl-lysine recognition site. C. The hydrophobic nature of the acetyl-lysine binding site is evident by the aromatic and hydrophobic residues that line the central acetyl-lysine-binding cavity, shown here on BRD4(1) (Figure adapted from Filippakopoulos *et al.* 2012 and Filippakopoulos & Knapp 2014).

associated factors (TAFs) (Venturini *et al.* 1999),

- transcriptional mediators (such as TAF1, also known as TAFII250) (Jacobson *et al.* 2000),

- nuclear scaffolding proteins (such as polybromo 1 (PBRM1, also known as PB1)) (Xue *et al.* 2000),

- the bromodomain and extra C-terminal domain (BET) family of proteins (Wu & Chiang. 2007; Brès *et al.* 2008; Muller *et al.* 2011).

BRD proteins mostly contain one or two BRDs, while some proteins, such as PB1, contain more than two BRDs (Lovén *et al.* 2013).

The presence of a BRD ensures appropriate targeting to specific K_{Ac} sites, where their accompanying domains govern the mechanism by which a particular BRD-containing protein will mediate gene regulation. Bromodomains have also been involved in binding to other acetylated proteins such as RelA (Huang *et al.* 2009), p53 (Barlev *et al.* 2001) and human immunodeficiency virus (HIV) Tat (Dorr *et al.* 2002).

3.2. Structure of bromodomains

The first BRD structure was determined by nuclear magnetic resonance (NMR) spectroscopy in 1999 (Dhalluin *et al.* 1999) and since then, the solution and/or crystal structures of over 40 BRDs have been determined (Vidler *et al.* 2012; Filippakopoulos *et al.* 2012).

Despite having large sequence variations, BRD modules share a conserved fold that comprises a left-handed bundle of four α helices (named αZ , αA , αB , and αC), linked by loop regions of variable length and charge (known as ZA and BC loops), which line the K_{Ac} binding site and determine binding specificity (Dhalluin *et al.* 1999; Filippakopoulos *et al.* 2012) (Figure 22A, B). The C- and N-termini are highly diverse and may comprise additional helices that extend the canonical BRD fold (e.g., the sixth BRD of PB1 has an additional C-terminal helix) or largely extended kinked helices that are present as C- or N-terminal extensions (for instance in TAF1L or ATAD2) (Filippakopoulos *et al.* 2012). Besides, cocrystal structures with peptides have demonstrated that K_{Ac} is recognized by a central deep hydrophobic cavity (Figure 22C), where it is anchored by a hydrogen bond to an asparagine residue (N140) presents in most BRDs (Dhalluin *et al.* 1999; Owen *et al.* 2000).

More recently, it has been demonstrated that some BRDs bind to two acetylated lysine histone marks that are simultaneously recognized by the same BRD module (Morinière *et al.* 2009). This property is shared by all members of the BET subclass of BRDs (Filippakopoulos *et al.* 2012).

The most notable structural difference within the BRD core fold is a hairpin insertion located between helix αZ and the long ZA loop which is present in all the members of the family VIII (Figure 21). Moreover, while the binding site is similar in all structures of BRDs, molecular dynamics calculations indicate that there are significant flexibility differences in the binding pocket (Steiner *et al.* 2013).

In contrast to the conserved fold of BRDs, their surface properties are highly diverse. The electrostatic potential of the surface area around the K_{Ac} binding site ranges from highly positively to strongly negatively charged, suggesting that BRDs recognize largely different sequences (Filippakopoulos *et al.* 2012).

Moreover, differences in the acetyl-binding pockets of BRD are important not only for the characterization of the BRD proteins but also for design of selective BRD inhibitors (Dhalluin *et al.* 1999; Filippakopoulos *et al.* 2012).

3.3. The BET family of proteins

3.3.1. The BET protein members

The BET proteins are a novel and small class of transcriptional regulators, all localize in the nucleus, and, as mentioned earlier, belonging to the subfamily II of BRD-containing proteins (Florence & Faller 2001). This subfamily is characterized by the presence of two BRD tandems (BD1, BD2), and the extra-terminal domain as outlined in details in the next paragraph. Additionally, in mouse and human, BET family is comprised of the three ubiquitously expressed and widely spread BRD2, BRD3 and BRD4, and the testis-specific form (BRDT), which is expressed specifically in testis, and ovary (Shang *et al.* 2004; Paillissan *et al.* 2007). *Drosophila melanogaster's fsh* (*female sterile homeotic (fsh)*), and *Saccharomyces cerevisiae's Bdf1* and *Bdf2* are corresponding homologs in other species (Wu & Chiang 2007).

Furthermore, BET proteins exist in two (i.e. long and short) isoforms generated by alternatively spliced transcripts differing at their 3' ends. The predominant long isoform of human and mouse BRD4 is often the only isoform detected (Dey *et al.* 2000; Shang *et al.* 2004; Devaiah & Singer 2012) and is responsible for almost all the biological functions attributed to BRD4 (Devaiah & Singer 2012), while the function of the short form (BRD4S, also known as HUNK1 in humans), if expressed (French *et al.* 2003), remained undefined (Wu & Chiang 2007; Devaiah & Singer 2012) until 2013 when Alsarraj *et al.* demonstrated that BRD4S is a nuclear membrane associated protein which interacts with metastasis susceptibility proteins such as signal-induced proliferation associated protein 1 (SIPA1) and ribosomal RNA processing 1 homolog B (RRP1B). This complex interacts with the inner nuclear protein SU2 (Sad1 and UNC84 containing 2) at the inner face of nuclear membrane and therefore plays a role in mechanotransduction of extracellular microenvironmental signal to the nucleus (Alsarraj *et al.* 2013). The genes encoding BET proteins are now designated *BRD2* (formerly referred to as *RING3* (really interesting new gene 3) or *Fshrg1* (female sterile homeotic related gene 1)), *BRD3* (also called *ORFX* or *Fshrg2*), *BRD4* (*Fsrg4*, *MCAP/HUNK1*), and *BRDT* (*Fsrg3*, *BRD6*). *BRD2* is a nuclear serine/threonine kinase possessing chromatin binding activity with preference for K12_{Ac} on histone H4 (Umehara *et al.* 2010) and transcription activity *via* its association with transcriptional regulators such as E2F1 (Denis *et al.* 2000; Kanno *et al.* 2004). *BRD3* and *BRDT* are less well characterized although mouse *Brdt* has been reported to induce global chromatin reorganization in an acetylation-dependent manner (Pivot-Pajot *et al.* 2003). *BRD4*, originally named *MCAP* (mitotic chromosome associated protein) (Dey *et al.* 2000), but also called *Fshrg4* or *Hunk1*, is a chromatin binding factor with preference for acetylated Lys14 (K14_{Ac}) on histone H3 and Lys5/12 on H4 (Dey *et al.* 2003). *BRD4* also shares strong structural similarity to the yeast BET proteins; *Bdf1* and *Bdf2* (Devaiah & Singer 2013). Additionally, the BRDs of *BRD4* bind to acetylated lysine 310 of *RelA*, the subunit of NF- κ B (Huang *et al.* 2009). In addition to their different affinity, BRDs of the BET family are known to have modest affinity for mono-acetylated lysine in a range of polypeptide contexts; however, their affinity is significantly greater when multiple acetylation sites exist within a span of one to five amino acids which occurs on histone tails and on certain transcription factors such

as GATA-1 and TWIST (Dey *et al.* 2003; Morinière *et al.* 2009; Lamonica *et al.* 2011; Filippakopoulos *et al.* 2012; Shi *et al.* 2014). In certain settings, this increase in affinity has been attributed to bidentate acetyl-lysine recognition that occurs within a single bromodomain pocket (Morinière *et al.* 2009; Gamsjaeger *et al.* 2011; Filippakopoulos & Knapp 2012). BET proteins can also bind to transcription factors in a BRD-independent manner to function as transcriptional cofactors (Wu *et al.* 2013).

Interestingly, the chromosomal locations of these *BRD* genes are adjacent to the four *Notch* genes found in the human genome with *BRD2* and *Notch4* on chromosome 6, *BRD3* and *Notch1* on chromosome 9, *BRD4* and *Notch 3* on chromosome 19, and *BRDT* and *Notch2* on chromosome 1 (Kasahara 1999; Houzelstein *et al.* 2002), indicating a likely functional relationship between human *BRD* and *Notch* gene families (Wu & Chiang 2007).

Retention on chromosomes during mitosis is likely a distinctive feature of the BET family (Chua & Roeder 1995; Dey *et al.* 2000; Dey *et al.* 2003; Kanno *et al.* 2004) because proteins of other BRD families are displaced from chromosomes during mitosis (Martínez-Balbás *et al.* 1995; Muchardt *et al.* 1996; Kruhlak *et al.* 2001). The presence of two BRD tandems may explain this unique feature of BET proteins as the deletion of bromodomain 1 (BD1) displaces BRD2 as well as BRD4 from mitotic chromosomes (Dey *et al.* 2003; Garcia-Gutierrez *et al.* 2012). Similarly, a double BRD2 mutant with point mutations Y152K and N428A in BD1 and BD2, respectively, previously shown to affect BRD2 functionality (Huang *et al.* 2007; Nakamura *et al.* 2007), has been reported to not associate to mitotic chromosomes (Garcia-Gutierrez *et al.* 2012). However, both BD1 and BD2 alone are not sufficient to promote association to mitotic chromosomes and other factors containing BRD tandems such as TAFII250 dissociate from mitotic chromosomes (Kruhlak *et al.* 2001). Nevertheless, the small conserved B motif has been identified as a dimerization domain involved in association to mitotic chromosomes and proper recognition of acetylated chromatin by BET proteins (Garcia-Gutierrez *et al.* 2012).

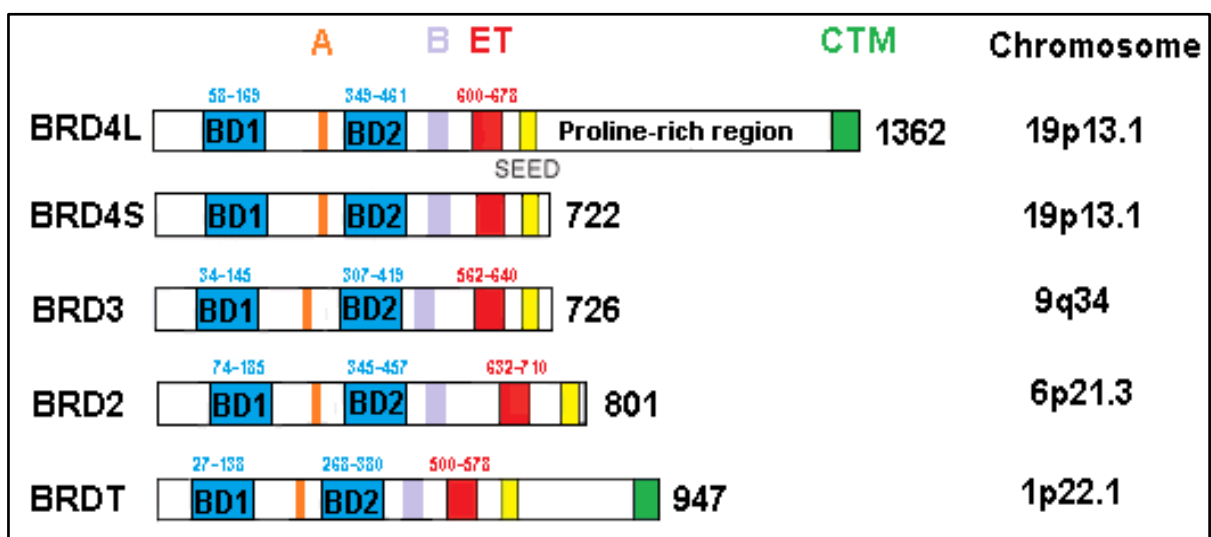


Figure 23: Domain architecture of BET proteins. BD1 and BD2 signify bromodomains. A, B and ET represent motif A, B and the ET domain, respectively. Colors indicate the different parts of BET proteins as mentioned in the text. Numbers indicate the amino acid boundaries of each domain or the corresponding amino acid residues of individual proteins (Figure adapted from Wu & Chiang 2007, Chung *et al.* 2011, Belkina & Denis 2012, and Fukazawa & Masumi 2012).

3.3.2. The BET domain architecture

The domain architecture of mammalian BET proteins is depicted in Figure 23. These proteins have a conserved modular architecture including two N-terminal tandem BRD modules (designated BD1 and BD2; responsible for acetyl-lysine recognition and shown in blue), an extra-terminal domain (ET; shown in red) on the C-terminal side of the double bromodomains (Florence & Faller 2001; Zeng & Zhou 2002; Mujtaba *et al.* 2007; Wu & Chiang 2007; Belkina & Denis 2012), several conserved motifs including (A, B motifs; shown in orange and light purple in Figure 26, respectively) as well as a serine–glutamate–aspartate-rich region (SEED; shown in yellow), and, in the case of BRDT and the long variant of BRD4 (BRD4L), a C-terminal motif (CTM; shown in green) (Abbate *et al.* 2006) that is sometimes referred to as PTEFb-interacting domain (PID) because it interacts with the positive transcription elongation factor b (PTEFb) (Wang & Filippakopoulos 2015). Although BD1, BD2, and the ET domains are characteristic of the BET family proteins, motifs B and SEED are also highly conserved among BET proteins. However, CTM (Abbate *et al.* 2006) and motif A (Paillisson *et al.* 2007) are not present in every protein.

A conserved region of 12 amino acids (KGVKRRKADTTTP), found at the end of the A motif between the two BET BRD modules was recently shown to act as a nuclear localisation signal and its deletion results in mislocalisation of the mutant proteins in HEK293T cells (Fukazawa & Masumi 2012).

While the function of BRDs has been well characterized, the function of other domains has been poorly investigated (Denis *et al.* 2000; Rahman *et al.* 2011). It is well established that BRDs of BET proteins bind to acetylated chromatin. While BRD2 seems to preferentially bind histone H4 at the mono-acetylated state (acetyl-K12), BRD4 and BRDT more efficiently recognize di-acetylated histone H4, at K5/K12 and K5/K8 positions, respectively (Dey *et al.* 2003; Kanno *et al.* 2004; LeRoy *et al.* 2008; Morinière *et al.* 2009; Sasaki *et al.* 2009; Ito *et al.* 2011). While many groups provided the evidences that the two isoforms of BRD4 (BRD4S and BRD4L) share the same N-terminal region except for the final three amino acids, have opposing effects in breast cancer growth and progression (Crawford *et al.* 2008; Alsarraj *et al.* 2011; Alsarraj *et al.* 2013) and reside in physically different subnuclear compartments (Alsarraj *et al.* 2013), Floyd *et al.* (Floyd *et al.* 2013) have shown that *BRD4*

encodes A, B and C splice isoforms with two BRDs and an ET domain presenting in each isoform. They demonstrated that the A isoform contains a CTD, which is notably absent in the B and C isoforms, and it is replaced by a divergent short 75 amino acid segment in the B isoform (Floyd *et al.* 2013). Moreover, despite possessing identical BRDs, BRD4S has been reported to have an expanded histone binding affinity relative to BRD4L (Alsarraj *et al.* 2013).

Bromodomain-mediated interactions with acetylated chromatin result in the localization of BET proteins to discrete locations along the chromosome, where they recruit other regulatory complexes to influence gene expression (Dey *et al.* 2003). The ET domain then serves as an interface to localize different complexes or proteins to chromatin. The identity of these associated factors may depend upon which modular domains are present. The ET domain may also interact with sequence-specific factors in order to help localize the BET protein to the active chromatin (Florence & Faller 2001).

Of BET family members, the function of BRD4 has been most thoroughly studied and will be the primary subject of the following paragraphs.

The structures of the BRD4 BD1 and BD2 have been solved and consist of 4 α -helices and 2 loops linking these α -helices (Vollmuth *et al.* 2009; Devaiah & Singer 2012). The BD1 primarily binds to H3 at K14_{Ac}, while the BD2 domain binds to H4 at K5_{Ac} and K16_{Ac} (Vollmuth *et al.* 2009; Devaiah & Singer 2012); that explains how BRD4 is retained on the chromatin during mitosis in a range of cell types. The solved ET domain structure consists of 3 α -helices and a loop (Vollmuth *et al.* 2009; Devaiah & Singer 2012).

3.4. Biological functions of BRD4 and BET proteins

3.4.1. BRD4 and BET proteins are mitotic bookmarks, and cell cycle regulators

Upon its binding to acetylated chromatin, BRD4 maintains the higher-order chromatin structure (Wang *et al.* 2012). Unlike non-BET BRD proteins (Dey *et al.* 2000; Umehara *et al.* 2010; Belkina & Denis 2012), BRD4 as well as BRD2 (LeRoy *et al.* 2008) remains bound to mitotic chromatin, therefore maintaining the epigenetic memory (Nishiyama *et al.* 2006; Yang *et al.* 2008; Belkina &

Denis 2012). Also, BRD4 is considered as an essential protein as *Brd4*^{-/-} embryos die shortly after implantation owing to mitotic defect (Dey *et al.* 2000; Maruyama *et al.* 2002; Dey *et al.* 2003; Belkina & Denis 2012). Such defects may occur owing the failure of Aurora B spindle checkpoint during mitosis, as Aurora B is BRD4 dependent (You *et al.* 2009; Belkina & Denis 2012). Furthermore, *Brd4* heterozygotes mice display severe pre- and postnatal growth defects as well as several anatomical abnormalities including head malformation, absence of subcutaneous fat, cataracts, and abnormal liver cells (Houzelstein *et al.* 2002). Moreover, knockdown of *Brd4* leads to a significant reduction in the growth of cultured cells (Wu *et al.* 2006) and embryonic stem cells do not grow in culture without its presence (Nishiyama *et al.* 2006). Interestingly, the inhibition of Brd4 causes a G2/M cell cycle arrest (Dey *et al.* 2000) and its overexpression in turn is associated with a G1/S arrest (Dey *et al.* 2003). Together these data reveal the role of BRD4 as a regulator of the cell cycle and cell growth at various stages. Similar to *Brd4*, knockdown of *Brd2* is lethal in mice (Gyuris *et al.* 2009; Shang *et al.* 2009; Wang *et al.* 2009), whereas no *Brd3* knockout model system is available (Belkina & Denis 2012). Furthermore, BRD4 remains associated with chromatin during mitosis, leading to rapid de-compaction of the surrounding chromatin and to post-mitotic transcription (Devaiah & Singer 2013). BRD4 functions as a positive transcriptional elongation factor complex (PTEFb) recruiter during interphase and as a mitotic bookmark responsible for accelerating post mitotic gene activation (Dey *et al.* 2009; Devaiah & Singer 2013). Indeed, BRD4 accelerates the dynamics of mRNA synthesis by de-compacting chromatin, facilitating transcriptional re-activation and marks the start sites of many M/G1 genes, and stimulates the expression of G1 genes by binding to multiple G1 gene promoters in a cell cycle-dependent manner and thus promotes cell cycle progression to S phase as shown using a small hairpin (sh) RNA approach (Mochizuki *et al.* 2008; Zhao *et al.* 2011). The *Brd4* shRNA vector stably knocked down Brd4 protein expression by about 90% in NIH3T3 cells and mouse embryonic fibroblasts and *Brd4* knockdown cells were found to be growth impaired and grew more slowly than control cells (Mochizuki *et al.* 2008). Also, BRD4 seems to be required for the G2 to M phase transition of the cell cycle because microinjection of BRD4-specific antibodies leads to cell cycle arrest (Belkina & Denis 2012).

Moreover, through binding to acetylated histone tails, BET proteins have been described as transcriptional activators with a prominent role in the control of cell cycle-associated genes. Brd4 has been involved in controlling expression of cyclin D1 and other G1-associated genes required for progression to the S phase (Mochizuki *et al.* 2008; Yang *et al.* 2008). Similarly, Brd2 has been shown to play a crucial role as cell cycle regulators of normal cells as it has been implicated in controlling the expression of cyclins A2 and D1 (Sinha *et al.* 2005; Denis *et al.* 2006; LeRoy *et al.* 2008; Belkina & Denis 2012). In fact, the key transcriptional regulators genes of S phase, E2F1 and E2F2, are associated with BRD2 multi-protein complexes (Belkina & Denis 2012). However, BRD3-dependent functional relationships with the cell cycle control machinery in normal cells are poorly understood (Belkina & Denis 2012; Fu *et al.* 2015), although there is some evidence that forced expression of *Brd3* in nasopharyngeal carcinoma (NPC) downregulates the RB-E2F pathway, which regulates the transition from G1 to S phase (Zhang *et al.* 2010; Belkina & Denis 2012).

3.4.2. BRD4 and BET proteins are protein scaffolds

In view of their role as protein scaffolds, BET family proteins were firstly detected as protein scaffolds for the recruitment of transcription factors and chromatin organizers required in transcription initiation and elongation. They recruit a variety of proteins to chromatin and transcription sites (Fu *et al.* 2015). BRD4 recruits PTEFb *via* its BD2 to site of active transcription and may be recruited by transcriptional mediator complexes independent of PTEFb (Wu & Chiang 2007). Additionally, the ET domain of BRD4 recruits independently transcription-modifying factors, including glioma tumor suppressor candidate region gene 1 (GLTSCR1); NSD3, a SET domain-containing histone methyltransferase; JMJD6, a histone arginine demethylase; and CHD4 (chromodomain helicase DNA-binding protein 4), a catalytic component of the NuRD nucleosome remodeling complex (Rahman *et al.* 2011; Liu *et al.* 2013). Different from BRD4, BRD2 binds to ϵ -aminoacetyl groups of nucleosomal histone lysines in a PTEFb- independent manner and, with related BRD proteins, it provides a scaffold on chromatin to recruit E2F proteins (Denis *et al.* 2000; Peng *et al.* 2007), HDACs (Denis *et al.* 2006), HAT (Sinha *et al.* 2005) and proteins involved in chromatin remodeling including SW1/SNF subunits

and elements of the mediator complex (Houzelstein *et al.* 2002; Belkina & Denis 2012), thereby coupling histone acetylation to transcription. Similar with BRD2, BRD3 may regulate cell processes through E2F-RB pathway. Moreover, it can directly recognized acetylated transcription factor, GATA1, which is essential for the targeting of GATA1 to chromatin (Vollmuth *et al.* 2009).

3.4.3. BRD4 and BET proteins function as transcriptional regulators

BET proteins play a key role in regulating the transcription of growth-promoting genes (Houzelstein *et al.* 2002). Besides binding acetylated chromatin *via* its BRDs, BRD4 interacts with nonhistone proteins to regulate transcription, DNA replication, cell cycle progression and other cellular activities (Wu & Chiang 2007). The role of BRD4 in transcriptional regulation was first suggested by the constituents of its associated protein complex including Mediator and PTEFb (Jiang *et al.* 1998; Jang *et al.* 2005; Yang *et al.* 2005; LeRoy *et al.* 2008; Garnier *et al.* 2014). Indeed, BRD4 was first identified as an interaction partner of mouse Mediator complex, a multiprotein coactivator that links transcription factors to polymerase II activation (Jiang *et al.* 1998). It was also found to associate with Mediator in human cells (Wu & Chiang 2007; Dawson *et al.* 2011). Moreover, BRD4 and Mediator occupy similar sites across the genome and can stabilize one another's occupancy at certain regions (Donner *et al.* 2010; Lovén *et al.* 2013).

The bromodomain protein BRD4 is one major regulator of PTEFb and stimulates RNA polymerase II-dependent transcription (Jang *et al.* 2005; Yang *et al.* 2005; Chen *et al.* 2014). In contrast to BRD2 and BRD3, BRD4 presents CTD which is essential for BRD4 association with PTEFb (Bisgrove *et al.* 2007). Indeed, tow regions of BRD4 bind directly to PTEFb: the CTD interacts with cyclin T1 and CDK9 while BD2 recognizes the acetylated region of cyclin T1 (Jang *et al.* 2005; Bisgrove *et al.* 2007; Schröder *et al.* 2012). These interactions result in preventing PTEFb from association with 7SK sn RNA/HEXIM1 (7SK small nuclear RNA/hexamethylene bis-acetamide inducible protein 1), a ribonucleoprotein complex that sequesters PTEFb in an inactive state (Jang *et al.* 2005; Yang *et al.* 2005), resulting in phosphorylation of the CTD of RNA polymerase II primarily at serine 2 (ser2) of its heptad (YSPTSPS) repeats and thereby facilitating productive mRNA transcript elongation (Price

2000; Garriga & Graña 2004; Nechaev & Adelman 2011; Devaiah & Singer 2012; Devaiah *et al.* 2012; Shi & Vakoc 2014). Notably, the interaction between BRD4 and PTEFb is particularly important for rapid transcriptional induction, as occurs at mitosis exit and in response to signal-dependent activation of DNA-binding transcription factors (Yang *et al.* 2008; Dey *et al.* 2009; Zippo *et al.* 2009). The recruitment of active PTEFb is crucial for the sustained presence of RNA polymerase II in active genes and for the transcription initiation and elongation. Indeed, PTEFb and RNA polymerase II are crucial for the expression of cell proliferation supporting genes, including c-MYC and its target genes (Rahl *et al.* 2010).

Furthermore, Devaiah *et al.* (Devaiah & Singer 2012; Devaiah *et al.* 2012) have reported BRD4 as an atypical RNA polymerase II ser2 kinase which directly phosphorylates the heptad repeats of RNA polymerase II at ser2 and remains associated with RNA polymerase II until productive elongation, suggesting additional mechanism through which BRD4 might directly regulate this elongation. However, the biochemical mechanism underlying that intrinsic kinase activity is to date unclear since BRD4 lacks homology to other known kinase domains (Shi & Vakoc 2014).

Moreover, the ET domain of BRD4 has been linked to transcriptional regulation independent of PTEFb by interacting with other chromatin regulators including the lysine methyltransferase NSD3 and the arginine demethylase JMJD6 (Rahman *et al.* 2011; Liu *et al.* 2013). The ET domain has been also found to associate with ATP-dependent nucleosome remodeling enzymes SW1/SNF and CHD4 (Rahman *et al.* 2011). These interactions imply that BRD4 alters chromatin structure at its occupied sites (Shi & Vakoc 2014).

3.5. Involvement of BET proteins in cancers and impact of BET bromodomain inhibitors

As key drivers of transcriptional events, BET proteins have been reported to be deregulated in a variety of diseases, such as viral infections, inflammation, obesity, metabolic disorders, autoimmune diseases, neurological disorders and cancers (for a review on the role of BET proteins in various diseases, see Filippakopoulos & Knapp 2014). Here, we will only present the present knowledge on

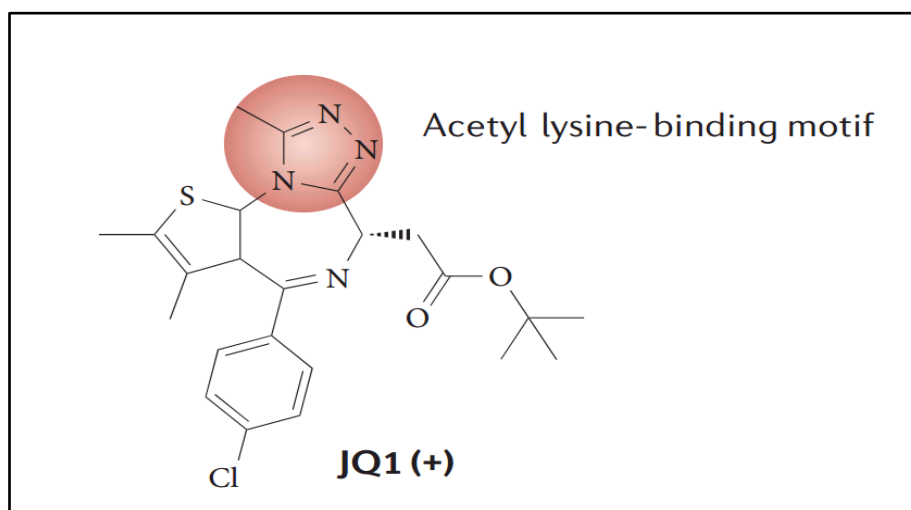


Figure 24: Structure of the small-molecule inhibitor of BRD4 protein, JQ1. The parts of the molecule that displace the ϵ -acetyl-lysine group of the histone are circled in red (Figure adapted from Belkina & Denis 2012).

the involvement of BET proteins, and particularly BRD4, in malignant diseases, since we have focused our work on this field.

3.5.1. Role of BET proteins in solid tumors

Accumulating evidences have revealed the involvement of BET family proteins in tumorigenesis (Kadota *et al.* 2009; Balkina & Denis 2012; Barbieri *et al.* 2013; Khochbin 2013; Zhang *et al.* 2015). The first clue linking BRD4 with tumor development was the finding of BRD4-NUT fusion oncogene in nuclear protein in testis (NUT) midline carcinoma (NMC), a rare and aggressive form of intrathoracic squamous carcinoma. Through the chromosomal rearrangement of *NUT* gene, BRD4 contributes to NMC development (Kubonichi *et al.* 1991; French *et al.* 2001; French *et al.* 2003; French 2010; French 2012). Of note, in the majority of the cases of NMCs, most of the coding sequence of NUT on chromosome 15q14 is fused with BRD4 creating chimeric genes that encode BRD4-NUT fusion protein which blocks differentiation and supports a high cell proliferation rate, while in the remaining cases, NUT is fused to BRD3 (French *et al.* 2008; French 2010). Also, the BRD4 inhibitor JQ1 treatment leads to terminal differentiation of BRD4–NUT-positive NMC cell lines and shows antitumor properties in mouse xenograft models of NMC (Filippakopoulos *et al.* 2010), leading to the initiation of clinical studies with I-BET762 (Shi & Vakoc 2014; Jung *et al.* 2015). Figure 24 shows the structure of JQ1 (Belkina & Denis 2012).

In addition to genomic rearrangement, BET proteins contribute to cancer development through other mechanisms as they are known to be deregulated in cancer (Belkina & Denis 2012; Wang & Filippakopoulos 2014; Chen *et al.* 2014; Zhou *et al.* 2015). In fact, BRD2, BRD3, and BRD4 proteins are upregulated in glioblastoma multiforme, the most common and malignant adult brain tumor (Pastori *et al.* 2014; Pastori *et al.* 2015). BRD4 is also found upregulated in in *Pkd1* mutant renal epithelial cells and tissues (Zhou *et al.* 2015). Recently, BRD2 has been shown to induce lung adenocarcinoma through its interaction with RUNX3 in a KRAS-dependent fashion (Lee *et al.* 2013). Besides, the expression of BRD4 has been found deregulated in breast cancer biopsies (Crawford *et al.* 2008), and in human colon cancer cell lines and primary tumors (Rodriguez *et al.* 2012). In addition,

the BRD4 transcriptional signature in breast cancer was found to discriminate rate of disease progression (Crawford *et al.* 2008).

Interestingly, the functional effects of BRD4 on metastasis and stem cell transformation have been recently determined to reside in the C terminus proline rich domain of the protein since deletion of this region results in epithelial-to-mesenchymal transition (EMT) and stem cell-like conversion (Alsarraj *et al.* 2011; Alsarraj & Hunter 2012). Very recently, BRD4 has been also found to promote both tumor growth and EMT in hepatocellular carcinoma (Zhang *et al.* 2015).

Furthermore, BRDT transcript has been identified as a cancer testis antigen in 25% of non-small cell lung cancer and 1 of 12 cases of both esophageal cancer and squamous cell carcinoma of the head and neck (Scanlan *et al.* 2000).

Notably, recent reports have uncovered BRD4 as a central regulator of estrogen receptor α (ER α) function and potential therapeutic target in ER α -dependent cancers like breast cancer (Nagarajan *et al.* 2014). In fact, BRD4 regulates estrogen receptor α (ER α)-induced gene expression by affecting elongation-associated phosphorylation of RNA polymerase II and histone H2B monoubiquitination in ER α -positive breast cancers. Of note, ER α controls cell proliferation and tumorigenesis by recruiting various cofactors to estrogen response elements (EREs) to control gene transcription such as cyclin D1 and MYC (Shang *et al.* 2000; Castro-Rivera *et al.* 2001). Consistently, BRD4 is required for proliferation of ER α -positive breast and endometrial cancer cells and uterine growth in mice (Nagarajan *et al.* 2014). Interestingly, BRD4 occupies transcriptional start sites and EREs upon estrogen stimulation and also regulates ER α -dependent eRNA production, which requires CDK9 (Hah *et al.* 2013; Nagarajan *et al.* 2014).

Moreover, the interaction with TWIST, a basic helix-loop-helix transcription factor controlling mesoderm formation during normal development and also (EMT) during cancer progression, implicates a role for BRD4 in the biology of epithelial cancers, which is possibly related to the therapeutic effects of BET inhibitors observed in xenograft models of breast cancer (Shi *et al.* 2014).

3.5.2. Role of BET proteins in hematopoietic malignancies

The constitutive expression of Brd2 in B cell progenitors in mouse models has been also shown to promote a B cell malignancy which has a proteomic signature similar to human diffuse large B cell lymphoma, suggesting that BRD2, in addition to BRD4, might be relevant to lymphoid neoplasms (Greenwald *et al.* 2004; Staudt & Dave 2005; Romesser *et al.* 2009; Delmore *et al.* 2011; Tolani *et al.* 2013).

In addition, using small hairpin RNA (shRNA) library targeting 243 known chromatin regulators, BRD4 has been identified as a required factor for the maintenance of acute myeloid leukemia (AML). Indeed, knockdown of BRD4 *in vitro* and *in vivo* results in a robust antileukemic activity against AML (Zuber *et al.* 2011; Blobel *et al.* 2011).

Importantly, the highly selective and recently reported BET bromodomain inhibitors including JQ1 (Filippakopoulos *et al.* 2010), I-BET151 (Nicodeme *et al.* 2010; Seal *et al.* 2012), and I-BET762 (Mirguet *et al.* 2013) competitively and selectively occupy the acetyl binding pockets of the closely related BRDs, resulting in release from active chromatin and the suppression of downstream signal transduction events to RNA Polymerase II (Filippakopoulos *et al.* 2010; Dawson *et al.* 2011; Delmore *et al.* 2011; Puissant *et al.* 2013; Lovén, *et al.* 2013). Numerous reports have thereby revealed the promising therapeutic potential of these inhibitors *in vitro* and *in vivo* in several hematopoietic malignancies including Burkitt's lymphoma, multiple myeloma, AML, and mixed-lineage leukemia (MLL) (Zuber *et al.* 2011; Dawson *et al.* 2011; Mertz *et al.* 2011; Delmore *et al.* 2011; Herrmann *et al.* 2012).

3.5.3. Role of BET inhibitors in solid tumors and hematopoietic malignancies

BET inhibitors exhibit remarkable preclinical effects in various somatic cancers, such as melanoma (Segura *et al.* 2013), glioblastoma and brain tumors (Wyce *et al.* 2013; Cheng *et al.* 2013; Pastori *et al.* 2015), breast cancer (Alsarraj & Hunter 2012; Stuhlmiller *et al.* 2015), liver cancer (Zhang *et al.* 2015), lung cancer (Lockwood *et al.* 2012; Lee *et al.* 2013; Lenhart *et al.* 2015. Tsai *et al.* 2015), colon cancer (Rodriguez *et al.* 2012), and prostate cancer (Wyce *et al.* 2013; Asangani *et al.* 2014).The

mode of action and efficacy of these inhibitors has been attributed mainly to their ability to modulate transcription elongation of essential genes involved in cell cycle and apoptosis such as c-MYC (v-myc myelocytomatosis viral oncogene homolog) and BCL-2 (Dawson *et al.* 2011; Mertz *et al.* 2011; Delmore *et al.* 2011; Jung *et al.* 2015). Indeed, JQ1 and other BET inhibitors result in preferential loss of Brd4 at super enhancers and consequent transcription elongation defects that preferentially impact genes with super enhancers, including the oncogene MYC (Lovén *et al.* 2013; Valent & Zuber 2014). Of note, MYC is known to be the main transcriptional target of BET proteins and contributes to the pathogenesis of certain cancer types.

To date, BET inhibitors have been reported to downregulate MYC expression in diverse tumors including myeloma (Delmore *et al.* 2011; Chaidos *et al.* 2014), Mantle Cell Lymphoma (Sun *et al.* 2015); Burkitt's lymphoma (Mertz *et al.* 2011), AML and MLL (Dawson *et al.* 2011; Zuber *et al.* 2011; Herrmann *et al.* 2012; Picaud *et al.* 2013; Fiskus *et al.* 2014), acute lymphoblastic leukemia (ALL), and T cell ALL (Ott *et al.* 2012; Da Costa *et al.* 2013; Roderick *et al.* 2014; Tan *et al.* 2015), medulloblastoma (Henssen *et al.* 2013; Bandopadhyay *et al.* 2014; Venkataraman *et al.* 2014), glioblastoma (Rajagopalan *et al.* 2014), lung adenocarcinomas (Lockwood *et al.* 2012; Tsai *et al.* 2015), lung cancer and non-small cell lung cancer (Shimamura *et al.* 2013), prostate cancer (Asangani *et al.* 2014), ovarian cancer (Baratta *et al.* 2015; Qiu *et al.* 2015), and skin cancers (Segura *et al.* 2013; Shao *et al.* 2014), as well as in estrogen receptor (ER) α -positive breast cancers (Bihani *et al.* 2015). The MYC isoform NMYC has also found to be downregulated in neuroblastoma by BET inhibitors (Puissant *et al.* 2013; Wyce *et al.* 2013; Shahbazi *et al.* 2014). However, MYC is not always the main transcriptional target of BET proteins (Lockwood *et al.* 2012; Cheng *et al.* 2013; Lenhart *et al.* 2015), suggesting the existence of additional, downstream targets of BET proteins and that MYC expression is driven by unique, tumor type-specific regulatory mechanisms (Wyspiańska *et al.* 2013; Kumar *et al.* 2015). For instance, *JAK2V617F*-driven AML line HEL (human erythroleukemic) displays sensitivity to JQ1 without a pronounced effect on MYC expression (Wyspiańska *et al.* 2013). Transcriptional profiling of these cells following BET inhibition shows prominent downregulation of LMO, a leukemogenic transcription coregulatory (Wyspiańska *et al.* 2013).

Furthermore, the anticancer effects of BET inhibitors may be mediated in MYC-independent manner through suppression of the NF κ B pathway. Beside its role in the immune system, NF κ B is well known as a heterodimeric transcription factor that enhances cancer cell proliferation and plays an important role in the pathogenesis of cancer (Hayden & Ghosh 2012). Treating cells with JQ1 disrupts the RelA/Brd4 interaction (Huang *et al.* 2009) and leads to proteasome-mediated RelA degradation, which may further suppress the NF κ B pathway (Zou *et al.* 2014).

Interestingly, targeting viral replication by BET inhibition could have therapeutic benefits in many virus-induced cancers. Through its binding to the HPV proteins indicated earlier (You *et al.* 2004; Schweiger *et al.* 2006), BRD4 has been reported to play a role in the initiation of viral DNA replication but not for continuing replication. Indeed, human papillomavirus 16 (HPV16) is causative in many human cancers, including cervical and head and neck cancers (in over than 90% of the cases, Parkin & Bray 2006) and BRD4 is implicated in the regulation of DNA replication by the two viral proteins, E1 and E2 (HPV19 E1-E2) which can be effectively disrupted after treatment with JQ1 (Gauson *et al.* 2015).

Similarly, in KSHV infection and Epstein-Barr-virus-associated cancers like the Burkitt's lymphomas, BRD4 is involved in the maintenance of viral infections *via* its interactions with viral encoded proteins (You *et al.* 2006; Lin *et al.* 2008), while in primary effusion lymphoma (PEL) which is an aggressive form of non Hodgkin's B cell lymphoma associated with infection by KSHV, JQ1 and I-BET151 exert their activities through genome-wide perturbation of MYC-dependent genes (Tolani *et al.* 2013).

Recently, BRD4 has been additionally identified directly linked to the Merkel cell polyomavirus (MCV) protein large T antigen (LT) which is found in 80% of Merkel cell carcinoma cases, a rare and highly aggressive neuroectodermal tumor arising from mechanoreceptor Merkel cells (Feng *et al.* 2008; Wang *et al.* 2012). Merkel cell polyomavirus is the first human polyomavirus to be definitively linked to cancer. BRD4 interacts and colocalizes with MCV large T antigen (LT)/replication origin complex in the nucleus and recruits replication factor C (RFC) to the viral replication sites (Wang *et al.* 2012).

Given that diverse transcriptional programmes are attenuated in a tissue-specific manner by BET

inhibitors (Roe *et al.* 2015), and preclinical targeting of BET proteins has had initial success in addition to the diverse and tissue-specific roles of individual BRDs, it will be challenging to target specific diseases without severe side effects. Despite the emerging preclinical and clinical evidences for therapeutic and promising benefits of BET inhibition (Chaidos *et al.* 2015; Yu *et al.* 2015), concerns have to be raised regarding their safety. For instance, upon targeting BET proteins, care must be taken to monitor the effects on super-enhancer-controlled stem cell specific transcription factors such as OCT4 and PRDM14, which control stem cell identity and cell fate decisions (Di Micco *et al.* 2014).

Moreover, BET inhibition strongly modulates the innate immune response and may increase the risk of infections when used clinically. As an instance, in mice infected with the bacterial pathogen *Listeria monocytogenes*, JQ1 strongly downregulated the expression of nitric oxide (NO) synthase 2, which reduced NO production and increased the susceptibility to develop colitis (Wienerroither *et al.* 2014).

More interestingly, recent findings have revealed a transcription-independent role of BRD4 in DNA damage response (DDR). In fact, BRD4 isoform B, the isoform lacking the PTEFb-interacting region as mentioned earlier, functions as an endogenous inhibitor of DDR signaling upon ionizing radiation by recruiting the condensin II chromatin remodeling complex to acetylated histones through BRD-mediated interactions. Consistent with these findings, knockdown or inhibition of BRD4 isoform B promotes cancer cell survival following ionizing radiation in association with increased H2AX phosphorylation, raising that induction of DDR signaling by BRD4 inhibitor might promote radioresistance (Floyd *et al.* 2013). A similar effect was previously reported for a C-terminal truncation BRDT, as outlined earlier, that also stimulates chromatin compaction (Pivot-Pajot *et al.* 2003), suggesting that a regulatory role in chromatin decompaction may be attributed to the CTM found on the longer BET variants (BRD4 and BRDT) (Wang & Filippakopoulos 2015).

RESEARCH OUTLINE

It is well established in the literature that human MC models described before 2014 have many limitations. As an instance, none of them has a functional FcεRI and a short doubling time. Thus there was still a need to develop a relevant, inexpensive and reproducible *in vitro* model representing at the best the human MCs in terms of the main morphological and biological properties. In addition, no fully relevant models of advanced SM harboring only the *KIT* D816V mutant, the most commonly mutant found in SM, were still available. In addition, no curative treatment can be offered to date to patients with advanced SM, for whom prognosis remains poor to date. Thus, the two major aims of our work were:

- 1) to establish an *in vitro* model representing morphologically and biologically the human MCs (ROSA^{KIT WT} cell line) and another *in vitro* model representing neoplastic human MCs (ROSA^{KIT D816V} cell line) mimicking at the best the phenotype of malignant cells found in patients with advanced SM.
- 2) to investigate the potential of the epigenetic reader BRD4 as a new therapeutic target in advanced SM (ASM and MCL). Thereafter, the goal of our work was to demonstrate that BRD4 inhibition alone or in combination with inhibitors of other relevant signaling pathways, might be a new and effective therapeutic approach to treat patients with advanced SM.

RESULTS

Publication 1. "A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection"

R Saleh*, G Wedeh*, H Herrmann, S Bibi, S Cerny-Reiterer, I Sadovnik, K Blatt, E Hadzijusufovic, S Jeanningros, C Blanc, M Legarff-Tavernier, E Chapiro, F Nguyen-Khac, F Subra, P Bonnemye, P Dubreuil, V Desplat, H Merle-Béral, M Willmann, T Rüllicke, P Valent, and M Arock. Blood. 2014 Jul 3;124(1):111-20.

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RESULTS

Publication 1. "A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection"

In this first article, we reported the establishment and characterization of a novel FcεRI-positive SCF-dependent human MC line, named ROSA^{KIT WT} cell line, from normal umbilical CB-derived CD34+ enriched mononuclear cells (MNCs). CD34+ cells were cultured in the presence of recombinant human SCF (rhSCF), IL-6 and IL-3 (only for the first week) as described (Arock *et al.* 2008). After 8 weeks, cells continued to grow in culture with a short doubling time (around 48 hours). The ROSA^{KIT WT} cells are round cells with a metachromatic cytoplasm and strongly positive for tryptase and KIT, but they are negative for chymase. They can differentiate terminally (MCTC) after their co-culture with a monolayer of stromal cells (MS-5 cells). The ROSA^{KIT WT} cells display a relatively mature MC phenotype as they express FcεRI receptor, KIT (CD117), CD33, CD4, CD9, CD203c, and CD300a, while they do not express CD2 and CD25. These cells are clonogenic and easily freezable. Moreover, the stimulation with IgE + anti-IgE leads to activation and degranulation of the cells. Thus, the ROSA^{KIT WT} cell line seems to be the best model available today for studying the mechanisms involved in the differentiation and activation of human MCs, as well as for high throughput screening of anti-allergic agents (Saleh, Wedeh *et al.* 2014).

We then transfected ROSA^{KIT WT} cells with a lentivirus coding for Green Fluorescent Protein (GFP) + *KIT* D816V. Then, GFP+ cells were sorted and cultured without SCF, giving rise to a SCF-independent cell line. The ROSA^{KIT D816V} cells display more mature phenotype than ROSA^{KIT WT} cells, with a significant proportion of spindle-shaped cells, which is a hallmark of neoplastic MCs in BM of patients with SM. Interestingly, we found that the responsiveness of ROSA^{KIT D816V} cells to IgE-dependent stimuli was weak compared with that of ROSA^{KIT WT} cells. Interestingly, we have also reported that the *KIT* D816V+ MCs obtained from patients with SM do not show increased FcεRI-dependent histamine release or CD63 upregulation. Moreover, ROSA^{KIT D816V} cells engraft efficiently NOD/SCID IL-2Rγ^{-/-} (NSG) mice, giving rise to an aggressive SM (ASM)-like disease. Of note, the NSG mice bearing ROSA^{KIT D816V}-derived tumors do not show mediator-related symptoms. Thus,

ROSA^{KIT D816V} cell line is a unique model of *KIT* D816V+ neoplastic MCs which could be used *in vitro* and *in vivo* for studies on the pathophysiology and treatment of SM (Saleh, Wedeh *et al.* 2014).

MYELOID NEOPLASIA

A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by *KIT* D816V transfection

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Key Points

- ROSA^{KIT WT} is a new human SCF-dependent FcεRI-positive mast cell line that converts to SCF-independence by KIT D816V-transfection.
- The FcεRI-positive ROSA^{KIT D816V} clone is a major tool for studying cellular aspects of mastocytosis and responses to targeted drugs.

In systemic mastocytosis (SM), clinical problems arise from factor-independent proliferation of mast cells (MCs) and the increased release of mediators by MCs, but no human cell line model for studying MC activation in the context of SM is available. We have created a stable stem cell factor (SCF)–dependent human MC line, ROSA^{KIT WT}, expressing a fully functional immunoglobulin E (IgE) receptor. Transfection with *KIT* D816V converted ROSA^{KIT WT} cells into an SCF-independent clone, ROSA^{KIT D816V}, which produced a mastocytosis-like disease in NSG mice. Although several signaling pathways were activated, ROSA^{KIT D816V} did not exhibit an increased, but did exhibit a decreased responsiveness to IgE-dependent stimuli. Moreover, NSG mice bearing ROSA^{KIT D816V}-derived tumors did not show mediator-related symptoms, and KIT D816V-positive MCs obtained from patients with SM did not show increased IgE-dependent histamine release or CD63 upregulation. Our data show that KIT D816V is a disease-propagating oncoprotein, but it does not activate MCs to release proinflammatory mediators, which may explain why mediator-related symptoms in SM occur preferentially in the context of a coexisting allergy. ROSA^{KIT D816V} may

provide a valuable tool for studying the pathogenesis of mastocytosis and should facilitate the development of novel drugs for treating SM patients. (*Blood*. 2014;124(1):111-120)

Introduction

Mast cells (MCs) are tissue-resident cells that play an important role in inflammatory and allergic reactions.¹ Two types of human MCs have been described: MC_T containing only tryptase, and MC_{TC} containing substantial amounts of tryptase and chymase.^{2,3} MC_T cells are mostly found in mucosa, and MC_{TC} cells are more commonly detected in the connective tissues.⁴⁻⁷ The development of tissue MCs from hematopoietic progenitors is regulated by stem cell factor (SCF), the ligand of KIT.⁸⁻¹⁰ MCs express large amounts of vasoactive and immunomodulatory mediators as well as high-affinity receptors for immunoglobulin E (IgE; FcεRI). As a result, MCs are potent effector cells of type I allergic reactions.¹¹⁻¹³ Moreover, MCs play a pivotal role in the

initiation and regulation of immune responses against various pathogens.¹⁴⁻¹⁷

The MC lineage can give rise to a distinct myeloid neoplasm, namely mastocytosis, characterized by MC expansion in one or more organ systems.¹⁸⁻²¹ In mastocytosis patients, clinical symptoms result from MC-derived mediators and from the destructive invasion of MCs into various organs.²² However, the exact mechanisms underlying MC activation in systemic mastocytosis (SM) remain unknown. In SM, the *KIT* D816V mutation in the catalytic domain of KIT is found in up to 90% of all patients.²³ This mutant initiates a number of signal transduction events and is thought to trigger abnormal MC activation in patients with SM. However, not all patients with SM suffer from these

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R.S. and G.W. contributed equally to this work as joint first authors.

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symptoms, even if their neoplastic MCs display *KIT* D816V. Other studies have shown that such mediator-related symptoms are seen especially in SM patients who suffer from a coexisting allergy.^{22,24-26}

Studies of the biology of human MCs have mainly been conducted by using MC lines or MCs obtained from primary cultures of CD34⁺ cells.²⁷ The only 3 human MC lines available so far are HMC-1,²⁸ LAD2,²⁹ and LUVA.³⁰ None of these cell lines coexpress *KIT* D816V and a functional IgE receptor. Here, we describe the establishment and characterization of a novel FcεRI-positive SCF-dependent human MC line, ROSA^{KIT WT}, and of a factor-independent subclone, ROSA^{KIT D816V}, produced by stably expressing *KIT* D816V in ROSA^{KIT WT} cells. ROSA^{KIT D816V} cells expressed the IgE receptor and engrafted NOD/SCID IL-2Rγ^{-/-} (NSG) mice. However, unexpectedly, the responsiveness of ROSA^{KIT D816V} cells to IgE-dependent stimuli was weak when compared with normal MCs, ROSA^{KIT WT} cells, or normal basophils.

Materials and methods

Establishment and characterization of a new human SCF-dependent MC line, ROSA^{KIT WT}

A sample of normal umbilical cord blood (CB) was collected after informed consent was given by the patient in September 2009 (Clinic Ambroise Paré, Bourg-la-Reine, France). Mononuclear cells were separated by density centrifugation (Ficoll-Hypaque; GE Healthcare). CD34⁺ cells were enriched by immunomagnetic sorting by using the MACS system (Miltenyi Biotec) and were cultured in the presence of recombinant human SCF (rhSCF) (80 ng/mL; R&D Systems) exactly as described.²⁷ After 8 weeks, cells continued to grow in culture with virtually all cells resembling MCs.

The complete coding sequences of the *KIT* gene expressed in ROSA cells were analyzed as previously described³¹ and were found to be normal (wild-type). The resulting SCF-dependent MC line was termed ROSA^{KIT WT}. This cell line was further transfected with *KIT* D816V, converting it into an SCF-independent clone, ROSA^{KIT D816V} (see supplemental Methods available on the *Blood* Web site).

Morphologic and immunocytologic characterization of the ROSA cell lines

The 2 cell lines were analyzed by microscopy on cytospin preparations stained with May-Grünwald Giemsa (MGG) or toluidine blue, or on MGG-stained smears produced from a cell pellet. Transmission electronic microscopy (TEM) was performed on ROSA^{KIT WT} cells by using standard techniques.³² Immunocytochemical staining was carried out on cytospin preparations of ROSA cells by using antibodies (Abs) against tryptase, chymase, and KIT (CD117) as described.³³

Analysis of the effects of various cytokines on ROSA cells

In the first set of experiments, ROSA^{KIT WT} or ROSA^{KIT D816V} cells (3×10^5 per milliliter in 96-well plates; 100 μL per well) were incubated in medium with or without rhSCF (80 ng/mL) at 37°C and 5% CO₂ for 4 days. On days 1, 2, 3, and 4, 10 μL of WST-1 (Water Soluble Tetrazolium salt-1; Roche Applied Science) were then added to the corresponding wells, and the cells were incubated for 3 additional hours at 37°C. The numbers of viable cells were measured by reading the absorbance at 570 nm using a Multiskan-MS plate reader (Thermo Labsystems). Because rhSCF has no significant (increasing) effects on the growth of ROSA^{KIT D816V} cells, we treated ROSA^{KIT WT} (cells with or without SCF) or ROSA^{KIT D816V} (cells without SCF) with various cytokines, each at 100 ng/mL: interleukin-3 (IL-3), IL-4, IL-6, IL-9, interferon gamma (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF), and nerve growth factor (NGF) (all from R&D Systems) at 37°C and 5%

CO₂ for 3 days. Then, 10 μL of WST-1 was added, and the cells were incubated for 3 additional hours at 37°C before the numbers of viable cells were measured.

Assays of cell activation and mediator release in ROSA cells

ROSA^{KIT WT} or ROSA^{KIT D816V} cells were first primed with recombinant human IL-4 (20 ng/mL; BioLegend) and human monoclonal IgE (2 μg/mL; Calbiochem) for 5 days to enhance the expression of FcεRI before stimulation.²⁷ To determine the expression of CD203c and β-hexosaminidase release, cells were incubated in control medium or medium containing anti-human IgE (5 μg/mL) (BioValley) or 1 μM of calcium ionophore (Cai) A23187 (Sigma) for 1 hour. Then, cells were stained by an allophycocyanin-conjugated anti-human CD203c Ab. The fluorescence intensity was measured by using a FACSCalibur flow cytometer (BD Biosciences). For the β-hexosaminidase release assay, stimulated cells were centrifuged, and β-hexosaminidase activity was measured by spectrophotometry in the supernatants and in cell lysates.³⁴ The percentage of β-hexosaminidase release was determined as described.³⁵ To determine histamine release, cells were incubated in control buffer or buffer containing mouse anti-human IgE (clone Depsilon2; Immunotech) (0.1 to 10 μg/mL) for 30 minutes at 37°C. Thereafter, cells were centrifuged, and the histamine content of the cell-free supernatants and the cell pellets was analyzed by radioimmunoassay (Immunotech). The percentage of histamine release was calculated as described.³⁶ To determine prostaglandin D₂ (PGD₂) and tumor necrosis factor α (TNF-α) release, cells were incubated in control medium, anti-IgE Ab (5 or 10 μg/mL), or Cai (1 μM) at 37°C for 1 hour (for PGD₂ release) or 6 hours (for TNF-α release) and then centrifuged. PGD₂ and TNF-α levels were determined in the supernatants by using a specific enzyme immunoassay for PGD₂ (Cayman Chemical) and a specific enzyme-linked immunosorbent assay for TNF-α (R&D systems) according to the manufacturers' instructions.

Western blotting

HMC-1.2 cells exhibiting 2 *KIT* point mutations, V560G and D816V (kindly provided by Dr J. H. Butterfield, Mayo Clinic, Rochester, MN), ROSA^{KIT WT} cells, and ROSA^{KIT D816V} cells were incubated in control medium or with rhSCF (80 ng/mL) for 5 minutes. Western blots were performed with Abs against total or phosphorylated STAT5 (all from Santa Cruz Biotechnology), total KIT, phosphorylated KIT, and total or phosphorylated AKT (all from Cell Signaling Technology). A mouse monoclonal IgG1 anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ab (Santa Cruz Biotechnology) was used as loading control.

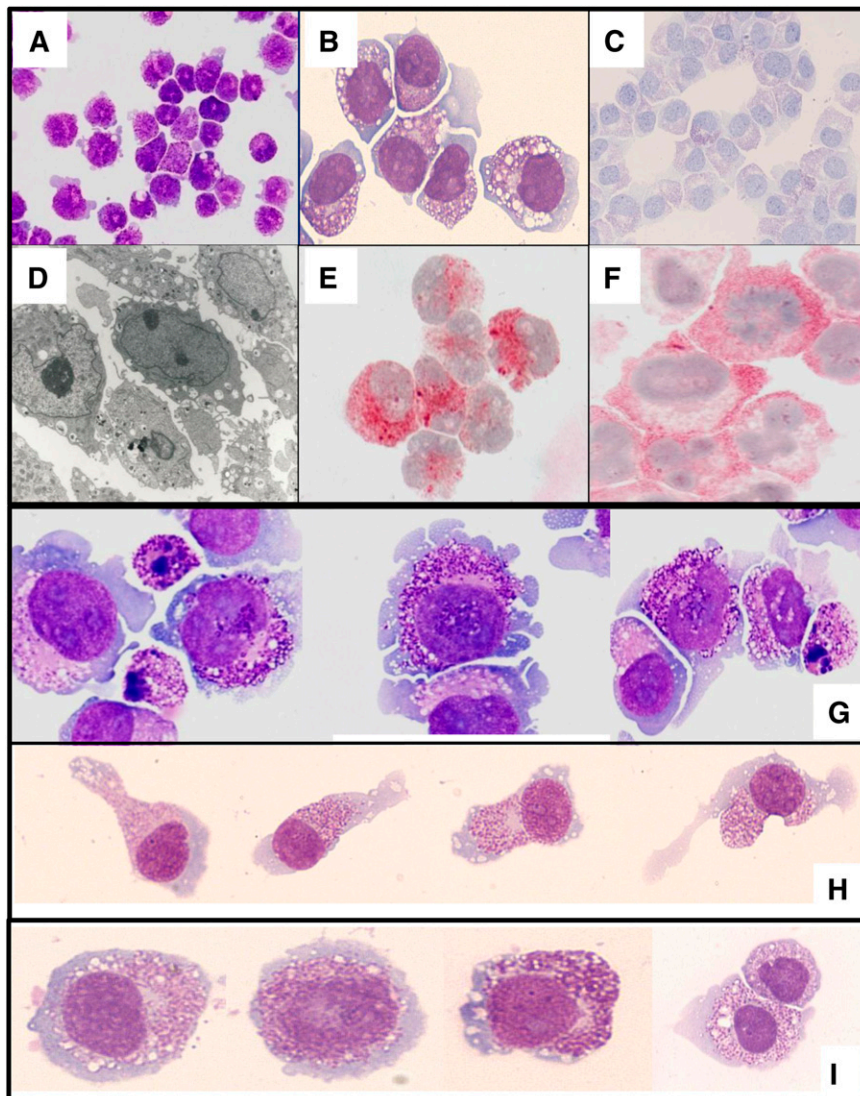
Assessment of the antiproliferative effects of tyrosine kinase inhibitors on ROSA cells

Cells (3×10^5 per milliliter; 100 μL per well) were incubated in 96-well plates with 1 μM of imatinib, 1 μM dasatinib (both supplied by Sequoia Research) or 1 μM midostaurin (Sigma) diluted in dimethylsulfoxide (DMSO; 0.1% final) or with DMSO alone (0.1%) in serum-free medium (with rhSCF at 80 ng/mL for ROSA^{KIT WT}) at 37°C (5% CO₂) for 48 hours. The number of viable cells was then determined in each condition as described in supplemental Methods.

Xenotransplantation of ROSA cells in NSG mice

ROSA^{KIT WT} or ROSA^{KIT D816V} cells (0.4 to 5×10^6) were either injected into the tail vein (intravenously) or transplanted under the skin (subcutaneously) bilaterally dorsocaudal of the scapula (3 to 5 mice per group) of adult NSG mice (The Jackson Laboratory). NSG mice were irradiated (2.4 Gy) 24 hours prior to intravenous injection. After injection, mice were inspected daily and euthanized as soon as disease-related symptoms appeared (intravenous group), the tumor grew to a maximum of 1 cm in diameter (subcutaneous group), or after a maximum observation period of 6 months. Bone marrow (BM) cells were obtained from flushed femurs, tibias, and humeri. Subcutaneous tumors were collected and dispersed by collagenase digestion. In addition, BM, lung, liver, spleen, and tumor samples were prepared for histologic and immunohistochemical analyses (supplemental Methods).

Figure 1. Phenotypic characterization of the ROSA cell lines. ROSA^{KIT^{WT}} cells were stained by (A-B) MGG or (C) toluidine blue. (D) The ultrastructure of ROSA^{KIT^{WT}} cells was analyzed by transmission electron microscopy. In addition, ROSA^{KIT^{WT}} cells were treated with an antibody against (E) tryptase or (F) KIT (CD117). Positivity was revealed by indirect immunocytochemistry as described in "Material and methods." (G) Cytospin preparations or (H-I) glass smears of ROSA^{KIT^{D816V}} cells were stained by MGG. (G) Note the presence of many granules in ROSA^{KIT^{D816V}} cells and of apoptotic cells, a phenomenon likely to be related to terminal maturation induced by the introduction of the *KIT* D816V mutant. (H) On glass smear, a significant proportion of the ROSA^{KIT^{D816V}} cells presented with spindle-shaped appearance, which was not noticed for (I) ROSA^{KIT^{WT}} cells. Magnification: A and C, $\times 200$; B, E, G, H, and I $\times 500$; D, $\times 2000$; F, $\times 1000$.



Flow cytometry analyses of ROSA cells

ROSA^{KIT^{WT}} cells and ROSA^{KIT^{D816V}} cells were analyzed by flow cytometry using a panel of monoclonal Abs directed against various leukocyte differentiation antigens (supplemental Table 1). In select experiments, xenotransplanted ROSA cells were quantified in BM samples and tumor dispersates obtained from NSG mice by flow cytometry as described³⁷ by using phycoerythrin-labeled monoclonal Abs directed against human CD117 (BioLegend) and CD45 (BD Biosciences). Engrafted ROSA cells were defined as CD45⁺/CD117⁺ cells (at least 0.1% of all nucleated cells) detectable in mouse BM cell suspensions.

Statistics

Unless otherwise stated, data represent the mean values \pm standard deviation of at least 3 independent experiments. When appropriate, data were analyzed by using the Student *t* test. Differences were considered as statistically significant if $P < .05$.

Animal studies

Animal studies were approved by the ethics committee of the Medical University of Vienna and were carried out in accordance with guidelines for animal care and protection and protocols approved by Austrian law (GZ

66.009/0040-II/10b/2009). This study was conducted in accordance with the Declaration of Helsinki.

Results

Establishment and growth characteristics of ROSA^{KIT^{WT}}, a new SCF-dependent human MC line, and of its SCF-independent subclone ROSA^{KIT^{D816V}}

ROSA^{KIT^{WT}} cells have a doubling time of 24 hours, and are split every 3 to 4 days. Optimal growth is obtained at 2×10^5 cells per milliliter in rhSCF (80 ng/mL). Because the *KIT* D816V mutant is commonly found in neoplastic MCs in patients with SM,³⁸ we transfected ROSA^{KIT^{WT}} cells with a lentiviral vector encoding *KIT* D816V. The resulting SCF-independent line, named ROSA^{KIT^{D816V}}, exhibited the same doubling time as ROSA^{KIT^{WT}} cells cultured with rhSCF (80 ng/mL). The two ROSA cell lines have been deposited to the Collection National de Cultures de Microorganismes (CNCM, Institut Pasteur, Paris), under the number CNCM I-4551 for the *KIT* WT cell line and CNCM I4552 for the *KIT* D816V⁺ cell line. In addition, the two ROSA cell lines have been patented (Patent WO/2013/064639).

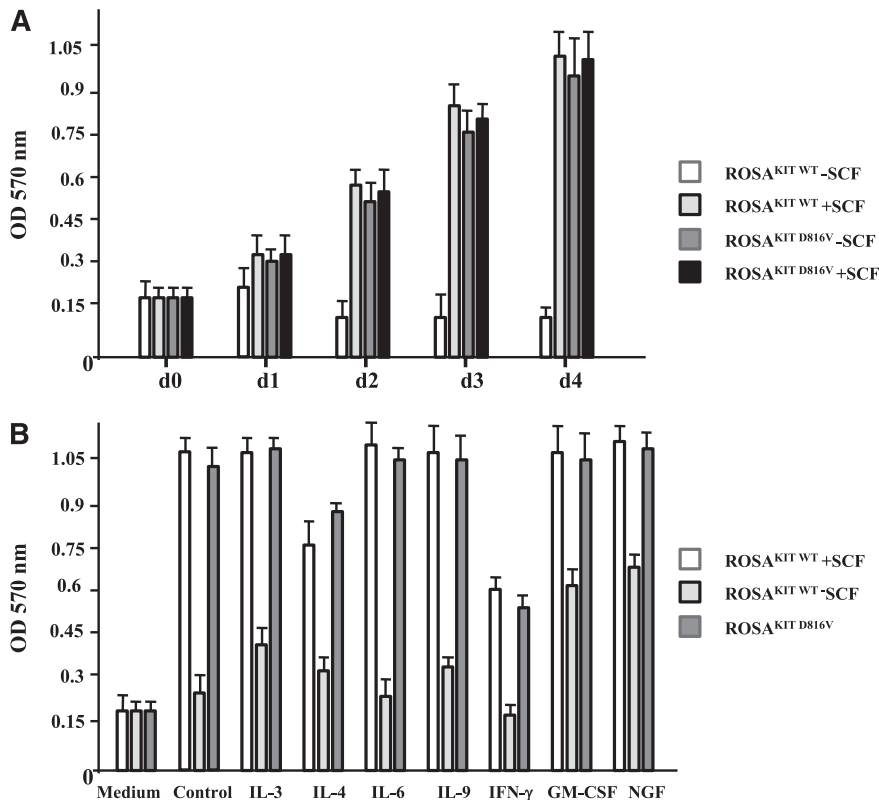


Figure 2. Effect of SCF and of various cytokines on the proliferation of the 2 different ROSA cell lines. (A) ROSA^{KIT} WT or ROSA^{KIT} D816V cells were treated or not treated with rhSCF (80 ng/mL) for 4 days at 37°C. At each time point, 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added in corresponding wells, and the cells were incubated for 3 additional hours in an incubator at 37°C. The number of living cells was then measured for each condition by reading the absorbance at 570 nm. (B) ROSA^{KIT} WT (with or without SCF) or ROSA^{KIT} D816V cells (without SCF) were treated with various cytokines (all at 100 ng/mL) for 3 days. At that time, 10 μ L of MTT was added in corresponding wells, and the cells were incubated for 3 additional hours in an incubator at 37°C. The number of living cells was then measured for each condition by reading the absorbance at 570 nm. Data presented are the mean \pm standard deviation (SD) from 3 independent experiments. OD, optical density.

ROSA^{KIT} WT and ROSA^{KIT} D816V cells are MCs by morphology, TEM, and phenotype

MGG-stained ROSA^{KIT} WT cells are round cells with a relatively high nuclear-to-cytoplasm ratio (Figure 1A-B). The slightly basophilic cytoplasm contained purple granules, their number and size being variable from cell to cell. Metachromasia was documented by staining with toluidine blue (Figure 1C). TEM studies showed that ROSA^{KIT} WT cells have a large nucleus with loose chromatin and 1 to 2 nucleoli (Figure 1D). The cytoplasm contained ribosomes, a well-developed Golgi apparatus, abundant mitochondria, and secretory granules with spherical electron-dense cores surrounded by electron-lucent space similar to those described in human CB-derived MCs (CBMCs).³⁹ In addition, almost all cells stained strongly positive for tryptase (Figure 1E) and KIT (Figure 1F) but were negative for chymase (data not shown).

Morphologic analysis of ROSA^{KIT} D816V cells showed significant differences when compared with ROSA^{KIT} WT cells. Indeed, ROSA^{KIT} D816V cells appear to be more granulated (Figure 1G). Interestingly, a significant percentage of ROSA^{KIT} D816V cells exhibited a spindle-shaped morphology on smears (Figure 1H), which was not observed with ROSA^{KIT} WT cells (Figure 1D). However, despite signs of maturation, ROSA^{KIT} D816V cells retained an MC_T phenotype because they expressed only tryptase but not chymase (data not shown).

Conventional cytogenetics and fluorescence in situ hybridization analyses of the ROSA cell lines

In initial cultures as well as after 12 months in culture, ROSA^{KIT} WT cells exhibited a complex karyotype, with a derivative chromosome 1 (der(1)inv(1)(p31q21)del(1)(q24q32)) and 2 subclones: one with a complete trisomy 5, and the other was predominant with a partial

trisomy 5 (+del(5)(q14q34)) (supplemental Figure 1A). Results from fluorescence in situ hybridization analysis confirmed the expression of aberrations observed by karyotyping in ROSA cells (supplemental Figure 1B-C). Interestingly, ROSA^{KIT} D816V cells exhibited the same karyotype after lentiviral infection (not shown).

Analysis of cell surface antigens in ROSA cells and responses to IL-4

Both ROSA cell lines have a comparable phenotype with a strong positivity for Fc ϵ RI α and KIT (CD117) (supplemental Table 2). The level of KIT (CD117) was higher in ROSA^{KIT} D816V cells compared with ROSA^{KIT} WT cells. A number of other antigens known to be expressed by human cultured MCs were also detected on ROSA cells, such as CD32, CD44, CD50, CD66a, CD166, CD203c, and CD300a (supplemental Table 2).⁴⁰ In addition, IL-4 induced a significant decrease in the expression of KIT as well as an increase in expression of Fc ϵ RI in both ROSA cell lines (supplemental Figure 2). These data strongly suggest that ROSA cells are MCs.

Response of ROSA^{KIT} WT and ROSA^{KIT} D816V cells to various cytokines

When comparing the effect of rhSCF (80 ng/mL) on the proliferation of ROSA^{KIT} WT cells and of ROSA^{KIT} D816V cells during a 4-day period, we observed that ROSA^{KIT} WT cells deprived of SCF were not able to grow, whereas ROSA^{KIT} WT cells cultured in the presence of SCF, as well as ROSA^{KIT} D816V cells cultured in the absence of SCF or presence of rhSCF, proliferated at an apparently similar rate (Figure 2A), suggesting that constitutive activation of the mutant KIT D816V is sufficient to induce optimal growth of ROSA cells.

We then investigated the potential effects of IL-3, IL-4, IL-6, IL-9, IFN- γ , GM-CSF, or NGF (all at 100 ng/mL) on spontaneous or

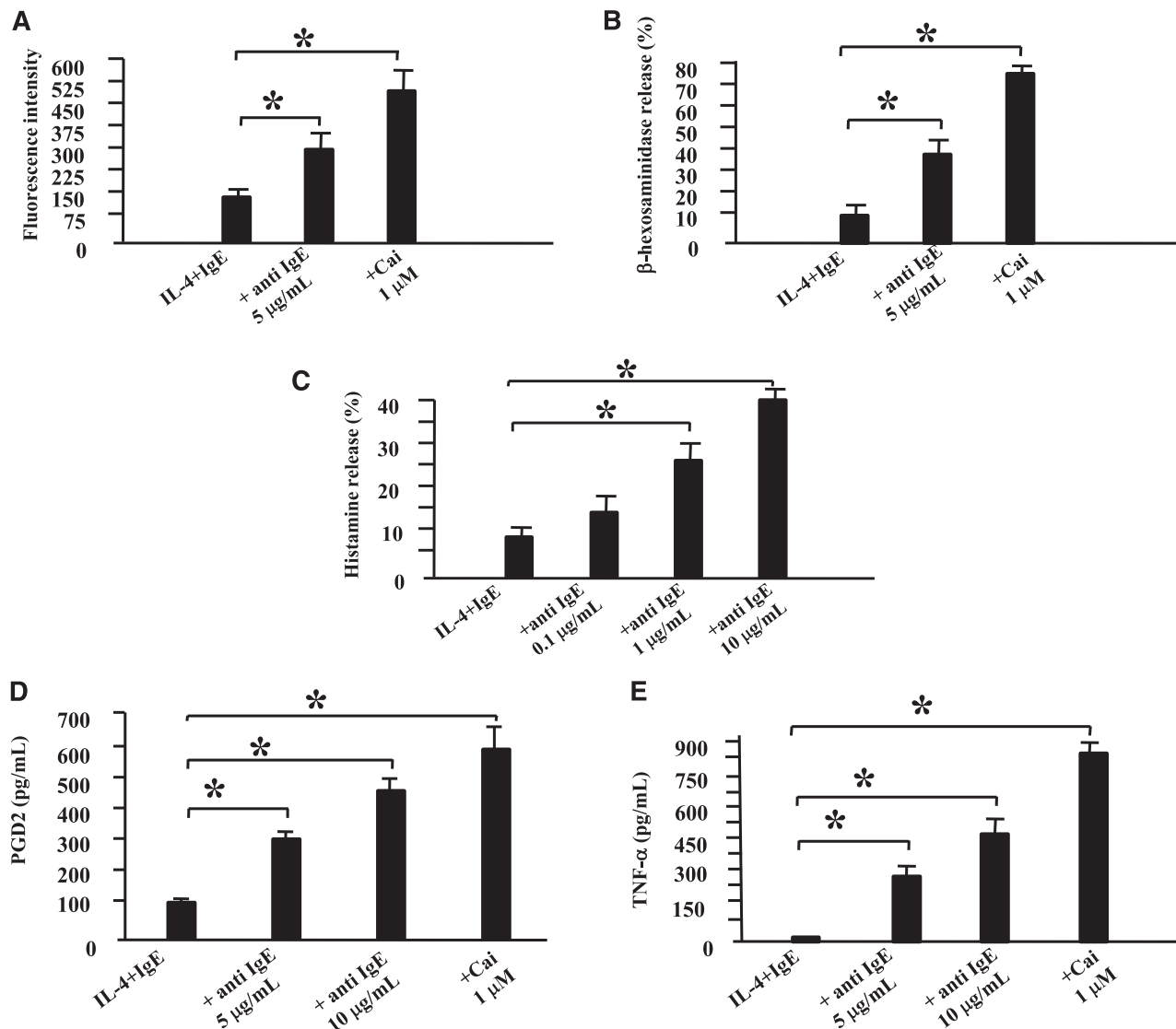


Figure 3. Biologic effects of the cross-linking of FcεRI on ROSA^{KIT} WT cells. ROSA^{KIT} WT cells were primed first with recombinant human IL-4 (recombinant human IL-4; 20 ng/mL) and human monoclonal IgE (2 μg/mL) for 5 days to increase the expression of FcεRI. (A) To determine CD203c upregulation, ROSA^{KIT} WT primed cells were stimulated by anti-human IgE (5 μg/mL) or by Ca-ionophore (Cai) (1 μM) for 1 hour. After labeling with an anti-human CD203c Ab coupled with allophycocyanin (APC), the fluorescence intensity was measured by using a FACSCalibur flow cytometer. (B) To determine β-hexosaminidase release, ROSA^{KIT} WT primed cells were stimulated by anti-human IgE or by Cai for 1 hour. β-hexosaminidase enzymatic activity was measured in the cell-free supernatants and after cell lysis as described in Materials and methods. (C) To determine histamine release, primed ROSA^{KIT} WT cells were stimulated with various concentrations of anti-human anti-IgE Ab at 37°C for 30 minutes. Thereafter, cells were centrifuged at 4°C, and the supernatants and cell pellets were analyzed for histamine content by using a specific radioimmunoassay kit. Results of histamine release are expressed as percentage of total histamine. (D) To examine synthesis of PGD2, primed cells were stimulated by anti-human IgE (5 or 10 μg/mL) or by Cai (1 μM) for 1 hour. Thereafter, cells were centrifuged, and the cell-free supernatants were analyzed for PGD2 content by using a specific enzyme immunoassay (EIA) kit. (E) To study the synthesis of TNF-α, primed ROSA^{KIT} WT cells were stimulated by anti-human IgE (5 or 10 μg/mL) or by Cai (1 μM) for 6 hours. Thereafter, cells were centrifuged, and the cell-free supernatants were analyzed for TNF-α content by using a specific EIA kit. For each parameter, data presented are the mean ± SD from 3 independent experiments. *Significantly different from the control (IL-4 + IgE) at $P < .05$.

SCF-driven proliferation of ROSA^{KIT} WT cells in the absence or presence of SCF (80 ng/mL) for 3 days. In addition, the same cytokines were added to ROSA^{KIT} D816V cells cultured without SCF. As shown in Figure 2B, NGF, and to a lesser extent IL-3 and GM-CSF, were able to partly rescue ROSA^{KIT} WT cells from cell death induced by SCF withdrawal. By contrast, none of the cytokines tested had any effect on increasing proliferation of ROSA^{KIT} WT cells in the presence of SCF or on the proliferation of ROSA^{KIT} D816V cells in the absence of SCF, except IL-4 and IFN-γ, which decreased the SCF-induced proliferation of ROSA^{KIT} WT cells and the autonomous proliferation of ROSA^{KIT} D816V cells (Figure 2B).

FcεRI cross-linked ROSA^{KIT} WT cells release MC mediators

Anti-IgE-induced activation of ROSA^{KIT} WT cells resulted in a significant upregulation of CD203c (Figure 3A), which was not the case neither for ROSA^{KIT} D816V cells, nor for purified neoplastic BM MCs from SM patients (supplemental Results and Figures 5 and 7). Corresponding results were obtained in a β-hexosaminidase-release assay. In fact, anti-IgE-activated ROSA^{KIT} WT cells released up to 38% of the enzyme after 1 hour (Figure 3B). As expected, calcium ionophore A23187 induced a strong increase in the expression of CD203c on ROSA^{KIT} WT cells (Figure 3A) as well as a release of up to 80% of β-hexosaminidase (Figure 3B), which is consistent with

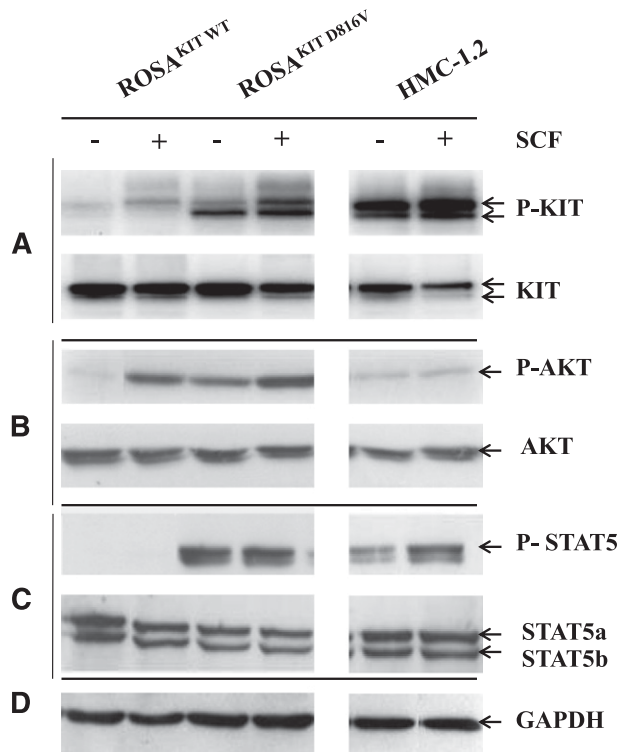


Figure 4. Detection of spontaneous phosphorylation of KIT, AKT, and STAT5 in ROSA^{KIT D816V} cells by western blotting. Cell lysates from unstimulated ROSA cell lines and from the HMC-1.2 cell line or from the same cells stimulated for 10 minutes with rhSCF were subjected to electrophoresis in sodium dodecyl sulfate polyacrylamide gel electrophoresis and treated with Abs against (A) anti-human total KIT or p-KIT Y719, (B) anti-human total AKT or p-AKT S473, or (C) anti-human total STAT5 or p-STAT5 Y694. (D) An anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The lanes were run on the same gel but were noncontiguous.

previous data.⁴¹ In addition, anti-IgE-induced activation of ROSA^{KIT WT} cells resulted in histamine release (Figure 3C). PGD2 and TNF- α were also synthesized and released by ROSA^{KIT WT} cells upon stimulation with anti-IgE or Cai A23187 (Figure 3D-E). Besides, the cellular histamine level averaged approximately 15 pg per cell in ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells, which is more than in primary CBMCs (4.5 pg per CBMC),⁴² LAD2 cells (3.1 pg per cell), or HMC-1 cells (0.9 pg per cell).²⁹

KIT phosphorylation and KIT-dependent signaling are constitutively activated in ROSA^{KIT D816V} cells

Western blotting was performed with anti-phospho-KIT Ab (Y703) on ROSA cells and on HMC-1.2 cells in the presence or absence of rhSCF. As shown in Figure 4A, in the absence of rhSCF, ROSA^{KIT D816V} and HMC-1.2 cells exhibited a constitutively phosphorylated KIT (p-KIT), whereas ROSA^{KIT WT} cells exhibited only low levels of p-KIT. Stimulation with rhSCF resulted in higher phosphorylation of KIT in ROSA^{KIT WT} cells and increased phosphorylation of KIT in ROSA^{KIT D816V} cells but not in HMC-1.2 cells (Figure 4A).

We also examined the phosphorylation of the serine residue 473 of AKT in the presence or absence of rhSCF in ROSA cells and in HMC-1.2 cells. In contrast to ROSA^{KIT WT} cells, which require the presence of SCF to express phospho-AKT, ROSA^{KIT D816V} cells exhibited a significant phosphorylation of AKT in the absence of rhSCF, which was even higher than in HMC-1.2 cells (Figure 4B).

Moreover, we demonstrated the constitutive phosphorylation of STAT5 in ROSA^{KIT D816V} cells but not in ROSA^{KIT WT} cells (even in

the presence of rhSCF; Figure 4C). Finally, rhSCF did not alter p-STAT5 levels in ROSA^{KIT D816V}, whereas it increased this expression in HMC-1.2 cells (Figure 4C). Anti-human glyceraldehyde-3-phosphate dehydrogenase demonstrated equal loading in all conditions (Figure 4D)

ROSA cells can be used as a major new model system for in vitro drug testing

We evaluated the inhibitory effects of three tyrosine kinase inhibitors, imatinib, dasatinib, and midostaurin (PKC412), each at 1 μ M, on the proliferation of ROSA cell lines. In line with published results,⁴³ ROSA^{KIT WT} cells were sensitive to imatinib, dasatinib, and midostaurin, whereas imatinib had no effects on ROSA^{KIT D816V} cells (Figure 5). As expected, dasatinib and midostaurin were found to inhibit the growth of ROSA^{KIT D816V} cells (Figure 5).

The 2 ROSA cell lines differentially engraft NSG mice

BM samples (mice injected intravenously) or dispersed tumors (mice injected subcutaneously) were analyzed for the presence of CD117⁺/CD45⁺ cells. Nearly all the mice injected intravenously with the 2 different types of ROSA cells developed a significant BM engraftment, with a mean percentage of CD117⁺/CD45⁺ cells significantly higher in the mice injected with ROSA^{KIT D816V} (52%) than in those injected with ROSA^{KIT WT} cells (2.62%) (Table 1). Of 5 mice injected subcutaneously with ROSA^{KIT WT} cells, 1 developed a subcutaneous tumor. This was similar to the other group, in which 1 of the 4 mice injected subcutaneously with ROSA^{KIT D816V} cells presented a subcutaneous tumor (Table 1). Interestingly, cytocentrifuged BM-engrafted ROSA^{KIT WT} cells stained with MGG kept a morphology comparable to the one observed in vitro, whereas BM-engrafted ROSA^{KIT D816V} cells exhibited spindle-shaped features resembling those of MCs from patients with KIT D816V-positive SM (Figure 6).⁴⁴ In addition, there was a highly significant correlation between the level of BM engraftment by CD117⁺/CD45⁺ cells and the level of tryptase-positive cells on BM biopsy (supplemental Figure 9). Finally, in line with previous data showing that the

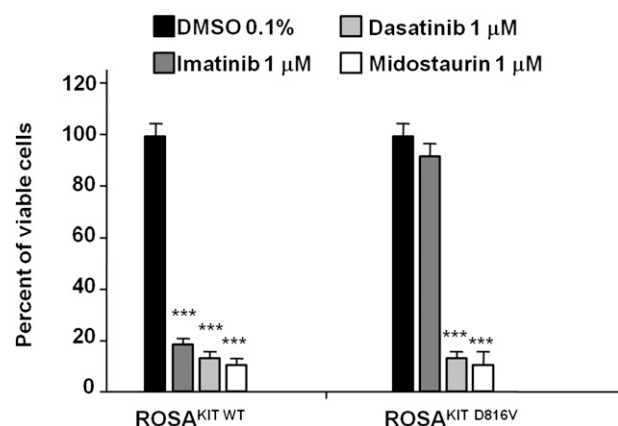


Figure 5. Effect of three tyrosine kinase inhibitors on the proliferation of the 2 ROSA cell lines. Cells were cultured for 48 hours in the presence of imatinib, dasatinib or midostaurin (1 μ M, provided in dimethylsulfoxide (DMSO) 0.1% final concentration) or in the presence of DMSO alone (0.1% final concentration) in the established culture medium (containing rhSCF at 80 ng/mL for ROSA^{KIT WT} cells but not for ROSA^{KIT D816V} cells). At the end of this incubation, 10 μ L of MTT was added in each well and the cells were incubated for 3 additional hours at 37°C. The number of living cells was then measured for each condition by reading the absorbance at 570 nm, and the results were expressed as a percentage of living cells compared with the control (DMSO 0.1% alone). Data presented are the mean \pm SD from 3 independent experiments. ***Significantly different from control (DMSO alone) at $P < .0001$.

Table 1. Overview of ROSA NSG experiments

	Cells injected × 10 ⁶	BM engraftment (positive/total)*	Median engraftment of intravenously injected mice (%)	Subcutaneous tumor growth (positive/total)†
ROSA ^{KIT} WT	5	4/5 (18 wk)	2.62	1/5
ROSA ^{KIT} D816V	0.4	4/4 (21 wk)	52	1/4

Table shows engraftment of ROSA^{KIT} WT and of ROSA^{KIT} D816V cells in NSG mice. Percentage of engrafted human MCs was assessed by flow cytometry using their double positivity for human CD45 and human CD117 antigens.

*Number of mice showing an engraftment of ROSA cells >0.1% in bone marrow after intravenous injection of total mice injected that survived more than 12 weeks.

†Number of mice showing a subcutaneous tumor formation after subcutaneous injection of total mice injected (2 sites per mouse) that survived more than 12 weeks.

microenvironment can influence MC phenotype,³ there was a clear tendency toward increased expression of chymase in subcutaneous tumors compared with BM-grafted cells (supplemental Table 5).

Discussion

MC research suffers from a lack of FcεRI-positive human MC cell lines established from normal hematopoietic progenitors. Here we

present a new tryptase-positive, chymase-negative, FcεRI-positive SCF-dependent human MC line, ROSA^{KIT} WT, derived from CD34⁺ cells of a normal human CB, with a normal KIT receptor. Their morphology and phenotype closely resemble those of primary cultured CBMCs.^{29,42,45} However, in contrast to CBMCs, ROSA^{KIT} WT cells can be cultured for years without loss of clonal stability, changes in phenotype, or significant alteration in their functional properties. Moreover, ROSA^{KIT} WT cells are easy to freeze and thaw by using conventional techniques (supplemental Methods). All these features suggest that ROSA cells represent a novel tool for MC research.

So far, only 2 FcεRI-positive human cell lines have been established: LAD2 and LUVA.^{29,30} These 2 cell lines have several limitations, such as a long doubling time for LAD2 or a weak expression of FcεRI by LUVA cells. Thus, the establishment of a ROSA^{KIT} WT cell line that has a short doubling time and stably expresses a functional FcεRI at high levels represents a new advanced tool for investigating human MC functions and for high-throughput screening of anti-allergic therapeutics.

KIT is a key receptor regulating the development of normal and neoplastic MCs, as evidenced by the presence of activating mutations in the *KIT* gene.²² In SM, the most frequent defect found, the *KIT* D816V point mutation, accounts for the pathophysiology of the disease.^{46,47} However, in vitro models available for studying the impact of *KIT* D816V mutation on human MCs are limited. Indeed,

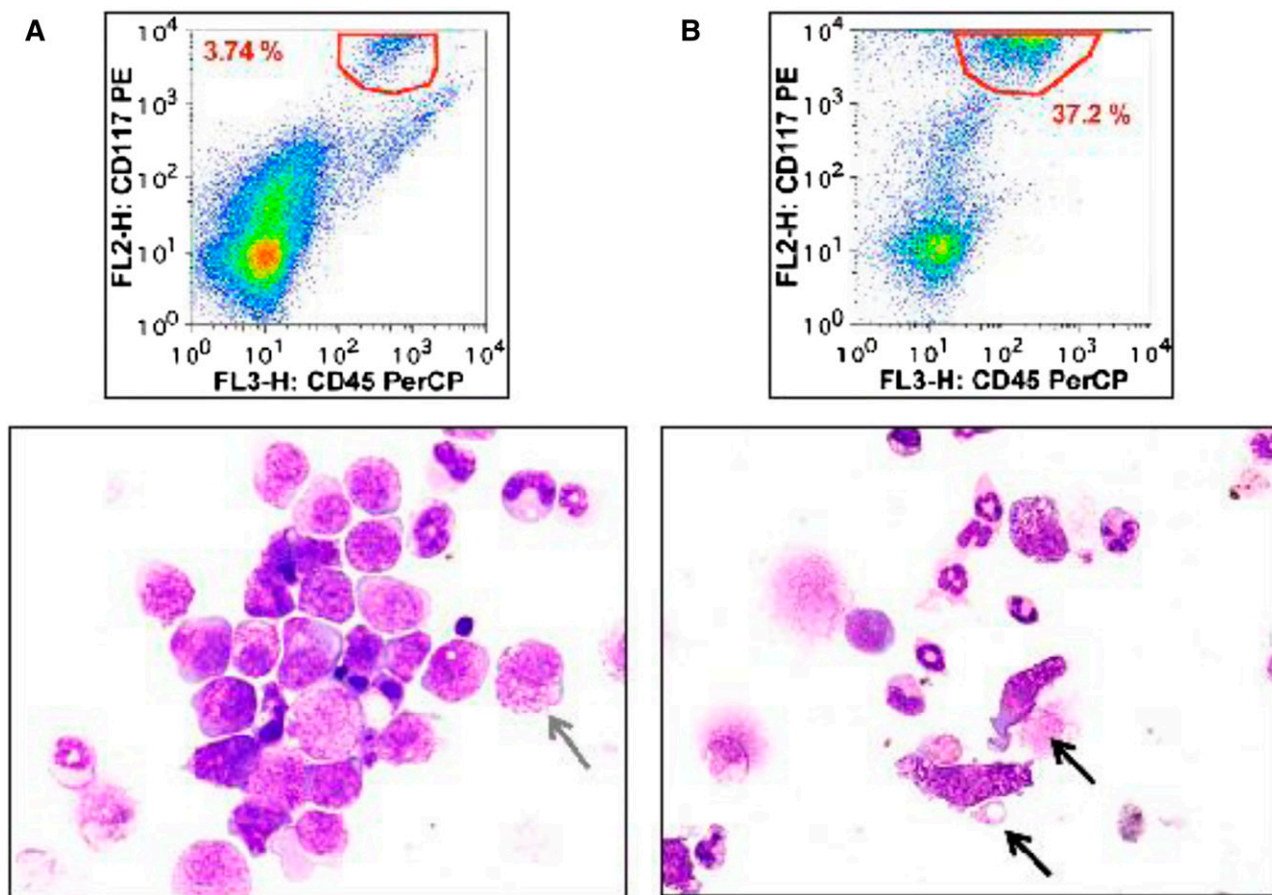


Figure 6. Morphologic appearance of BM-engrafted ROSA^{KIT} WT and ROSA^{KIT} D816V cells in NSG mice. Whole BM cells from NSG mice engrafted intravenously with (A) ROSA^{KIT} WT or (B) ROSA^{KIT} D816V cells were analyzed for their content of CD117⁺/CD45⁺ engrafted human MCs (A and B, top panels). The cells were also cytocentrifuged on glass slides, stained with MGG and then observed by using light microscopy (bottom panels). Note the presence of spindle-shaped MCs with an abnormal distribution of granules in the BM of mice engrafted with ROSA^{KIT} D816V cells (B, black arrows), whereas the BM MCs from mice engrafted with ROSA^{KIT} WT cells presented with a rounded shape, similar to the one observed in culture (A, gray arrows). Magnification is ×200. FL2-H, FL2 peak emission values; PerCP, peridinin chlorophyll.

the only model available is the HMC-1 cell line and its 2 subclones HMC-1.1 and HMC-1.2.⁴⁸ Both clones contain the *KIT* V560G mutation, whereas HMC-1.2 additionally exhibits the *KIT* D816V mutation.⁴⁸ Unfortunately, HMC-1 cells do not bear the *KIT* D816V mutation alone, and there is no HMC-1 subclone with a normal *KIT* gene, making it difficult to compare signaling pathways activated by normal or mutant forms of *KIT* in the same cell model. To overcome these limitations, we established the *KIT* D816V-positive SCF-independent ROSA^{KIT D816V} MC line, which constitutes the first in vitro model of human MC with *KIT* D816V mutant alone and for which a *KIT* WT equivalent exists.

ROSA^{KIT D816V} cells express CD117, CD203c, and tryptase but they also express FcεRI at a level comparable to that of ROSA^{KIT WT} cells. Interestingly, ROSA^{KIT D816V} cells could not be activated by anti-IgE in the same way as ROSA^{KIT WT} cells (data presented in supplemental Results and in Figure 5). These discrepant results led us to investigate whether malignant MCs from different subcategories of *KIT* D816V-positive SM were also insensitive to such activation. Interestingly, anti-IgE stimulated by BM MCs from such patients released little or no histamine and did not upregulate the activation marker CD63, and these data are presented in supplemental Results and in supplemental Figures 5 and 7. So far, the mechanisms underlying this phenomenon remain unknown. A decrease in expression of IgE receptors or other molecules relevant to MC activation in *KIT* D816V-transformed cells was excluded in our experiments. However, Itoh et al⁴⁹ have recently shown that MCs chronically exposed to SCF display a marked attenuation of FcεRI-mediated degranulation and cytokine production. Such desensitization may also play a role in *KIT* D816V-positive cells in which the *KIT* receptor is chronically phosphorylated. Since FcεRI and *KIT* use common signaling pathways, cross-desensitization may occur.⁵⁰ Alternatively, *KIT* D816V may induce the expression of negative regulators of MC activation, such as phosphatases. All in all, these findings might explain why many patients with SM, even with high MC burden, do not suffer at all from IgE-related mediator-related symptoms. Nevertheless, a number of patients may suffer from mediator-release symptoms related to non-IgE triggers, such as drugs, infection, or alcohol.⁵¹

Activation of *KIT* recruits a number of signaling pathways in MCs. Specifically, activation of the PI3-K/AKT pathway was shown to contribute to *KIT*-dependent proliferation, survival, maturation, adhesion, or activation.⁵² Interestingly, the introduction of *KIT* D816V in ROSA cells induced the constitutive activation of AKT, which is in line with previously reported data.⁵³ In addition, *KIT* D816V recruits alternative signaling pathways not evoked by *KIT* WT, particularly STAT5.⁵⁴⁻⁵⁶ Interestingly, ROSA^{KIT D816V} cells constitutively expressed p-STAT5, suggesting that they use the same alternative pathways as MCs in mastocytosis patients. Moreover, using inhibitors of AKT or STAT5, we showed that ROSA^{KIT D816V} cells are more sensitive to their antiproliferative effects than the ROSA^{KIT WT} cells (supplemental Methods, Results, and Figure 8). This is consistent with a previous report showing that (1) oncogenic *KIT* controls neoplastic MC growth through a STAT5/PI3-kinase signaling complex activating AKT and (2) knockdown of AKT or STAT5 activity inhibits growth of neoplastic *KIT* D816V-positive MCs.⁴⁷ To the best of our knowledge, this is the first demonstration that de novo introduction of the *KIT* D816V mutation leads to constitutive phosphorylation of STAT5 in a model of human neoplastic MCs. Thus, ROSA^{KIT D816V} cells represent valuable tools for exploring the mechanisms involved in mutant *KIT*-related signaling pathways and provide a robust model for screening potential STAT5 inhibitors. Moreover, in line with previously

reported data,^{57,58} ROSA^{KIT WT} cells, but not ROSA^{KIT D816V} cells, were sensitive to imatinib. Of note, ROSA cell lines were equally sensitive to dasatinib and midostaurin, which is also consistent with previous reports.^{59,60} Thus, the 2 ROSA cell lines seem to be suitable models for in vitro screening of selective inhibitors of the different forms of *KIT*.

Mastocytosis research suffers from a lack of animal models mimicking human SM. Indeed in the 2 models already published, which were obtained by using mice transgenic for mutant *KIT* in which the transgenes were selectively expressed in MCs,^{46,61} the targeted cells in both cases were murine MCs. Here, we show that human neoplastic *KIT* D816V-positive MCs can engraft at a high rate in NSG mice, not only in the BM, but also in spleen and lung, giving rise to a disease mimicking, at best, the situation encountered in SM patients. Indeed, as evidenced by immunohistochemistry for human tryptase on BM sections of NSG mice injected intravenously with ROSA^{KIT D816V} cells, compact infiltrates of tryptase-positive human MCs can be seen. Together with the spindle-shaped appearance of the neoplastic MCs found on BM smears of the animals, and with their positivity for *KIT* D816V, our animal model exhibits several features resembling SM in humans,⁶² making it the most similar model for human SM described so far.

In summary, we have established a new human FcεRI-positive MC line exhibiting the major characteristics of normal MCs, with several advantages over available human MC models in vitro, which might support future studies on the biology of MCs and on their pharmacologic modulation. In addition, we have derived a unique model of human MCs that expresses the *KIT* D816V mutant producing a mastocytosis-like disease in NSG mice. These noteworthy features should facilitate the development of new pathogenetic concepts and the testing of new anti-neoplastic drug therapies.

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Authorship

Contribution: M.A. and P.V. designed the experiments; R.S., G.W., H.H., S.C.-R., I.S., K.B., E.H., S.B., S.J., C.B., E.C., F.S., and P.B. performed the experiments; M.A., M.L.-T., F.N.-K., H.M.-B., M.W., T.R., and P.V. analyzed the data; P.D. and V.D. contributed essential reagents; M.A. and P.V. wrote the paper; and all authors approved this manuscript.

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A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by *KIT* D816V transfection

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Supplemental Methods, Results, Tables and Figures

R. Saleh *et al.* A new human mast cell line expressing a functional IgE receptor converts to factor-independence and tumorigenicity by *KIT* D816V-transfection

Supplemental Methods

Lentiviral transfection of ROSA^{KIT WT} cells with *KIT* D816V

Human *KIT* cDNA was cloned in pRRL lentiviral vector.¹ Production of lentivirus encoding *KIT* D816V and infection of ROSA^{KIT WT} cells were performed exactly as previously described.² The efficiency of infection was measured by flow cytometry (detection of green fluorescence of GFP) after 4 days. The GFP+ cells were selected by cell sorting and placed immediately in SFM deprived of rhSCF. The cells were maintained in culture for more than 2 years by regular dilution (every 3 or 4 days) in SFM without rhSCF. The resulting human autonomous MC line was termed ROSA^{KIT D816V}.

KIT-sequencing

Total RNA was extracted from 5.10⁶ ROSA *KIT* WT cells or from the same number of ROSA *KIT* D816V cells, using an RNeasy Mini Kit (Qiagen S.A.). RNA was reverse transcribed into cDNA using a StrataScript first-strand synthesis system (Stratagene) and random hexamer primers in a total volume of 25 μ l, according to the manufacturer's instructions. Next, the *KIT* coding sequence was amplified from 2.5 μ l of cDNA by PCR, using HotStarTaq DNA polymerase (Qiagen S.A.) and the primers already published by us³ for 40 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 45 seconds. The PCR products were purified using a GeneClean III *KIT* (Qbiogene), and the entire *KIT* coding region was directly sequenced using a BigDye terminator v1.1 kit (Applied Biosystems), the

sequencing primers already published,³ and an ABI Prism 3100 sequencer (Applied Biosystems). By these means, no mutations in *KIT* were found in ROSA^{KIT WT} cells whereas, as expected, ROSA^{KIT D816V} cells exhibited two variants of the *KIT* gene at position 816: GAC (WT) and GTC (D816V) (data not shown).

Quantitative PCR (qPCR) detection of KIT WT and KIT D816V mRNA

Total RNA from 5.10⁶ ROSA^{KIT WT} and ROSA^{KIT D816V}, or HMC1.2 as a control, was isolated using TRIzol (Life Technology) following exactly the manufacturer's protocol. RNA concentration and purity was determined using a Nanovue plus spectrophotometer (GE Healthcare) and samples were stored at -20°C until use. First strand cDNA synthesis was conducted using the iScript Select cDNA synthesis kit (Biorad) according to the manufacturer's instructions.

Real time PCR was performed using a LightCycler 1.5 Instrument (Roche) using LightCycler[®] FastStart DNA Master^{PLUS} SYBR Green I (Roche). Primer sequences used were designed as described by Kristensen *et al.*⁴: KIT WT F; a common forward primer 5'-AGAGACTTGFCAGCCAGAAAA-3', a reverse primer 5'-TTAACCACATAATTAGAATCATTCTTGATCA-3' for KIT D816V, a reverse primer 5'-TGCAGGACTGTCAAGCAGAG-3' for KIT WT. Reactions were compared to the levels of expression of mRNA for Hypoxanthine Phosphoribotransferase (HPRT) amplified using the following primers: HPRT F; 5'-ATGGACAGGACTGAACGTCTTGC-3'; and HPRT R; 5'-GACACAAACATGATTCAAATCCCTGA-3'. Real time PCR reactions were carried out in duplicate using the following cycle conditions: 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, at 60 °C for 1 min and at 72°C for 1 min. All qPCR experiments included a no template control (water). A serial dilution of the cDNA mixture was used to produce a standard curve and the cDNA of HMC1.2 served as a calibrator for gene expression analysis. The mRNA expression of KIT WT and KIT D816V in

ROSA cells was calculated relative to the expression of these genes in control HMC1.2, according to the delta-delta C_t method ($2^{-\Delta\Delta C_t}$).

Karyotyping and Fluorescence in situ Hybridization of the ROSA cell lines

Chromosomes were prepared after 24-48 hours of *in vitro* culture. Conventional R-banding analysis was performed with standard methods and karyotypes were defined according to the International System for Human Cytogenetic Nomenclature (ISCN 2013). Fluorescence *in situ* hybridization (FISH) analyses on metaphases were performed with commercially available probes, according to the manufacturer's protocols. The following probes provided by Kreatech (Amsterdam, Netherlands) were used: EGR1(5q31)/CSF1R(5q33)/hTERT(5p15) and whole chromosome painting (WCP)1. Results were recorded using Eclipse 90i fluorescence microscope (Nikon) fitted with appropriate filters, CCD camera and digital imaging software from Alphelys (Genikon).

Analysis of selected surface markers expressed on ROSA cell lines by flow cytometry

The expression of cell surface antigens on ROSA^{KIT WT} and ROSA^{KIT D816V} cells was analyzed by direct immunofluorescence using FACSCalibur and Aria flow cytometer (BD Biosciences) and the characteristics of the various antibodies used are provided in supplementary Table S1. Briefly, 2x10⁵ untreated cells were incubated with monoclonal murine anti-human antibodies specific for FcεRIα, KIT (CD117), CD2, CD3, CD4, CD5, CD8, CD9, CD10, CD11b, CD25, CD34, CD54, CD203c (all from Biolegend), CD13, CD14, CD16, CD17, CD19, CD20, CD22, CD23, CD24, CD30, CD31, CD32, CD33, CD38, CD44, CD45, CD48, CD50, CD52, CD56, CD59, CD63, CD64, CD66a, CD69, CD71, CD87, CD90, CD95, CD96, CD105, CD114, CD115, CD116, CD123, CD127, CD133, CD134, CD138, CD144, CD146, CD150, CD164, CD166, CD213a, CD218a, CD300a, and CD309 (all from BD Biosciences). The expression of FcεRIα and KIT was also determined before or after incubation of

the cell lines with rhIL-4 (20 ng/mL) (R&D) and human monoclonal IgE (2 μ g/mL) (Calbiochem) for 5 days. Isotype controls were used for each experiment, and at least 10,000 events were recorded for each analysis on a BD FACSCalibur and BD FACSAria.

Analysis of the clonogenicity of ROSA cells

Five hundred cells were plated in 35 mm Petri dishes containing 1 mL methylcellulose based medium (Methocult, Stem Cell technologies), supplemented with 20% fetal calf serum (FCS; PAA) and 0.3% bovine serum albumin (PAA) and with human rhSCF at 80 ng/mL for ROSA^{KIT WT} but not for ROSA^{KIT D816V} cells. Petri dishes were then placed in an incubator at 37°C and 5% of CO₂. Colonies of more than 50 cells were enumerated by microscopy after 15 days of culture.

Cryopreservation

ROSA cell lines were frozen and stored according to conventional freezing methods by suspension of cells at 10⁷/mL in a freezing medium made by 90% FCS (PAA) and 10% of DMSO (Sigma), samples being cooled immediately at -80°C overnight then kept in liquid nitrogen until used.

Analysis of the effect of withdrawal of SCF on the apoptosis of ROSA^{KIT WT} cells

ROSA KIT WT cells were seeded at 2x10⁵ cells per mL in flasks, in the absence or presence of recombinant human SCF(80 ng/mL) and incubated at 37°C and 5% CO₂ for 7 days (d0 to d7). At d0, d2, d4 and d7, an aliquot (10⁵ cells) was harvested for each condition, transferred in flow cytometry tubes, washed with cold PBS and treated by 10 μ g/mL Annexin V-APC (BD Biosciences) and 5 μ g/ml propidium iodide (PI, Biolegend) in 100 μ L incubation buffer (PBS supplemented with 1% FCS) for 15 minutes at RT in the dark and then analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Analysis of the effect of non-IgE triggers on the activation of ROSA cell lines

ROSA^{KIT WT} or ROSA^{KIT D816V} cells were incubated in control medium or medium containing peptidoglycan purified from *S. aureus* (PGN) (1, 5 or 10 µg/mL; Sigma), compound 48/80 (1, 5 or 10 µg/mL; Sigma) or 1 µM of calcium ionophore A23187 (Sigma) for 1 hour. Then, cells were stained by an anti-human CD203c antibody (Ab) (Biolegend) coupled with APC. The fluorescence intensity was measured using a FACSCalibur (BD Biosciences). For β-hexosaminidase release assay, stimulated cells were centrifuged and β-hexosaminidase activity was measured by spectrophotometry in the supernatants and in cell lysates.⁵ The percentage of β-hexosaminidase release was determined as described.⁶

Histamine release assay on cells from mastocytosis patients and from healthy volunteers

Major demographic and biological characteristics of mastocytosis patients involved in the study are detailed in Table S3. The histamine release assay was performed on dextran-enriched basophils (healthy donors, n=3), BM derived mononuclear cells from patients with SM (3 ISM, 2 ASM, 2 MCL; n=7) and PB derived mononuclear cells isolated from 2 ISM patients and 2 ASM patients (n=4) essentially as described.^{7,8} Before challenge with anti-IgE, cells were preincubated or not with 200 ng/ml human recombinant stem cell factor (SCF) (PeproTek). After incubation for 90 minutes, cells (1 x 10⁶/ml) were washed, and thereafter incubated with various concentrations of anti-IgE antibody E124.2.8 (Immunotech) (0.001-10 µg/ml) in histamine release buffer (HRB) (Immunotech) at 37°C for 30 minutes. Cells were then centrifuged at 4°C, and the cell-free supernatants and total suspensions recovered and analyzed for histamine content by radioimmunoassay (RIA, Immunotech). Histamine release was calculated as percent of total (cellular + extracellular) histamine. For primary cells derived

from patients with different categories of systemic mastocytosis, only three concentrations of anti-IgE antibody E124.2.8 (0.1, 1 and 10 μ M) were applied to induce histamine liberation.

Antibody staining and flow cytometry analysis of CD63 on stimulated BM MCs

To examine the effects of anti-IgE and C5a on expression of activation-linked cell surface antigens on bone marrow (BM)-derived mast cells (MCs), flow cytometry experiments were performed. Unseparated BM cells from *KIT* D816V+ SM patients (ISM, n=3; ASM/MCL, n=3) (ISM patients were patients N° 10, 11 and 12 presented in Table S3; ASM/MCL patients were patients N° 1, 4 and 9 of the same table; all these 6 patients having a *KIT* point mutation at position 816) and from normal/reactive bone marrow (n=1) were incubated with anti-IgE mAb E-124.2.8 (Beckman Coulter, Fullerton, CA) (10 μ g/ml), recombinant human C5a (Sigma-Aldrich, St. Louis, MO) (100 nM) or control buffer at 37°C for 90 minutes. Thereafter, cells were washed and stained with FITC-labeled CD63 mAb CLB-gran12 (Beckman Coulter), PE-Cy-7-labeled CD117 mAb 104D2 (ebioscience, San Diego, CA), APC-Cy7-labeled CD45 mAb HI30, and Pacific Blue-labeled CD34 mAb 581 (Biolegend, San Diego, CA) for 15 minutes at room temperature. After erythrocyte lysis with FACS-lysis solution (BD Biosciences, San Jose, CA), expression of cell surface antigens was quantified by multi-color flow cytometry on a FACSCantoII (BD Biosciences). MCs were identified as CD45+/CD117+/CD34- cells. Anti-IgE-induced and C5a-induced upregulation of CD63 on MCs was calculated from mean fluorescence intensities (MFI) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells and was expressed as stimulation index = SI (MFI_{stim}/MFI_{control}).

Analysis of the effects of AKT and STAT5 inhibitors on cell proliferation

In order to evaluate the impact of AKT or of STAT5 on proliferation, cells were seeded at a starting concentration of 3×10^5 per mL in 96-well plates (100 μ L per well) and incubated at 37°C and 5% CO₂ for 48 hours in the presence of various concentrations (0.01 to 5 μ M) of a selective inhibitor of AKT1/AKT2 activity (compound A6730; Sigma), or of a selective inhibitor of STAT5 (pimozide: 1 to 6 μ M; Sigma), both diluted in DMSO 0.1% final concentration, or in the presence of DMSO alone (0.1%) in SFM (with rhSCF at 80 ng/mL for ROSA^{KIT WT} but not for ROSA^{KIT D816V} cells). Ten μ L of WST-1 (Roche Applied Science) were then added in each well and the cells were incubated for 3 hours at 37° C. The number of living cells was then measured for each condition by reading the absorbance at 570 nm using a plate reader Multiskan-MS (Thermo-Labsystems).

Immunohistochemistry (IHC) in mouse tissue sections

IHC was performed on slides prepared from paraffin-embedded, formalin-fixed BM, lung, liver, spleen and skin tumor biopsy specimens. The indirect immunoperoxidase staining technique was performed according to established protocols using the anti-chymase mAb B7 (work dilution: 1:50) and the anti-tryptase mAb G3 (1:100) (both from Chemicon).^{9,10} Prior to staining, sections were pretreated with citrate buffer and microwave oven (tryptase stain) or proteinase and waterbath (chymase stain). Endogenous peroxidase was blocked by methanol/H₂O₂. Slides were incubated with primary mAb diluted in 0.05 M Tris-buffered saline (TBS) (pH 7.5) plus 10% FCS for 20 hours at 4°C then washed in TBS. After incubation with biotinylated anti-mouse IgG (Vector) for 30 minutes, slides were washed three times in TBS, and then exposed to Vectastain ABC KIT for 30 minutes at room temperature. 3-amino-9-ethylcarbazole (AEC) (Sigma) was used as chromogen. All slides were counterstained in Mayer's Hemalun solution.

Supplemental Results

Clonogenicity of the ROSA cell lines

In semi-solid medium cultures, clonogenic capability of ROSA^{KIT WT} cells (in the presence of rhSCF) was found to be around 7% (mean±SD: 6.8±1.7%), while ROSA^{KIT D816V} cells (in absence of rhSCF) appeared to be less clonogenic (mean±SD: 4.08±1.7%), which is consistent with their more differentiated state.

Withdrawal of SCF on ROSA^{KIT WT} cells induces rapid cell apoptosis

In a first set of experiments, we investigated the effect of the complete withdrawal of SCF on the survival of ROSA^{KIT WT} cells. For this purpose, ROSA^{KIT WT} cells were placed in their usual medium of culture, containing or not SCF (at the optimal predetermined concentration of 80 ng/mL), then incubated for 7 days. At day 0, day 2, day 4 and day 7, an aliquot of the cells placed in each condition was harvested and double labeled with Annexin-VAPC and propidium iodide (PI) in order to determine the percentage of apoptotic cells by flow cytometry. As shown in Figure S3, withdrawal of SCF induced a time-dependent increase in the percentage of apoptotic cells, which rose up to 40% at day 7, whereas the percent of apoptotic cells remained very low (around 5%) whatever the time of treatment when the cells were cultured in the continuous presence of SCF. In addition, a visual examination of the cultures at day 7 under an inverted microscope with phase contrast revealed that nearly 100% of the cells deprived of SCF appeared dead (most of the cells appeared as grey elements in this condition).

ROSA^{KIT D816V} cells express equivalent amounts of mRNA coding for *KIT* WT and *KIT* D816V

Since ROSA^{KIT D816V} cell line was derived from ROSA^{KIT WT} cells (which only express *KIT* WT gene) by lentiviral transfection of a construct coding for the *KIT* D816V gene, we decided to measure the

level of transcription of both genes into mRNA coding for KIT WT and for KIT D816V in this mutant cell line. We thus analyzed by quantitative RT-PCR (qRT-PCR) the amount of each type of mRNA, using for this purpose primers specific for KIT WT mRNA, as well as primers specific for KIT D816V mRNA. In addition, in the same experiments, we compared the expression of KIT WT mRNA in ROSA^{KIT WT} cells and in ROSA^{KIT D816V} cells. The results of the expression of each mRNA were calculated relative to the expression of these mRNA in HMC1.2 cells. As reported in Figure S4, the relative level of mRNAs coding for KIT WT was similar in ROSA^{KIT WT} cells and in ROSA^{KIT D816V} cells, which was expected since the mutant cell line can be considered as a daughter cell line derived from the ROSA^{KIT WT} cell line. In addition, ROSA^{KIT D816V} expressed only slightly more (1.2x) mRNA coding for the KIT D816V receptor than mRNA coding for the wild-type receptor (Figure S4), which is not significantly different and witnesses that both variants of KIT were quite equally expressed (at least at the mRNA level) in ROSA^{KIT D816V} cells. Finally, also as expected, ROSA^{KIT WT} cells did not express mRNA coding for the mutant KIT receptor (Figure S4).

ROSA^{KIT D816V} cells and primary KIT D816V+ mast cells exhibit constitutively activated KIT, but show a largely reduced or absent response to anti-IgE antibody

Although expressing the FcεRI at the same level than ROSA^{KIT WT} cells, ROSA^{KIT D816V} cells did not release histamine (Figure S5A), β-hexosaminidase, TNF-α or PGD2 after activation by anti-IgE (data not shown). At the same time, ROSA^{KIT D816V} cells were able to up-regulate CD203c and to release up to 80% of their β-hexosaminidase following stimulation with calcium ionophore. Interestingly, neoplastic KIT D816V+ BM MCs obtained from patients with ISM (Figure S5B), ASM or MCL (Figure S5C) released only little if any histamine when stimulated through the FcεRI. This failure to respond to IgE-induced activation was not related to an intrinsic defect in FcεRI, since (non-clonal)

basophils obtained from the same patients could be fully activated through the FcεRI (Figure S5D). Moreover, basophils derived from healthy donors were activated and released substantial amounts of histamine under the same conditions (Figure S5E).

ROSA cell lines respond differentially to activation by non-IgE triggers

Contrasting to ROSA^{KIT^{WT}} cells (Figure 3), we observed that ROSA^{KIT^{D816V}} cells were unresponsive to IgE-mediated activation (Figure S5A). For this reason, we sought to investigate whether this lack of response of the mutant cell line was limited to FcεRI triggering or was a general phenomenon of hyporesponsiveness towards other, non-IgE triggers. For this purpose, we stimulated ROSA^{KIT^{WT}} cells and ROSA^{KIT^{D816V}} cells for 1 hour with different concentrations of peptidoglycan purified from *S. aureus* (PGN), which is known to activate human MCs through TLR2,¹¹ or of compound 48/80, a cationic polymer inducing degranulation of human MCs.¹² We then analyzed the level of expression by these cells of the activation marker, CD203c, and compared it to the one of unstimulated cells or of cells stimulated with 1 μM of calcium ionophore A23187. In addition, we measured the percentage of β-hexosaminidase released by the two cell lines stimulated similarly. As reported in Figure S6, the level of stimulation evoked by a treatment with increasing concentrations of peptidoglycan (PGN) or compound 48/80, each used at 1, 5 or 10 μg/mL appears dose-dependent in ROSA^{KIT^{WT}} cells for up-regulation of CD203c as measured by flow cytometry (Figure S6A), as well as for β-hexosaminidase release (Figure S6C). By contrast, it appears that those two triggers are not able to induce activation at the same level for ROSA^{KIT^{D816V}} cells. Indeed, in ROSA^{KIT^{WT}} cells, PGN and compound 48/80 induced respectively up to 35 and 55% of mean β-hexosaminidase release at the optimal dose of 10 μg/mL, whereas in ROSA^{KIT^{D816V}}, the same triggers, used at the same dose, induced respectively only a mean β-hexosaminidase release of 20% and 22% (Figure S6B). The same differences can be noticed

for up-regulation of CD203c (Figure S6D). Although there is a trend to a tendency for ROSA^{KIT D816V} cells to increase their β -hexosaminidase release and to up-regulate CD203c with increasing concentrations of triggers, the values obtained with PGN or compound 48/80 used at 10 μ g/mL were not found statistically different (at $p < 0.05$) than the one obtained for control, unstimulated cells. These data suggest that the presence of the activating KIT D816V mutation seems to be also able to desensitize human MCs towards non-IgE triggers. The mechanism of this desensitization remains unknown, although one cannot exclude that, as for IgE-triggering, a mechanism of competition for a number of intracellular common substrates might exist, due to the presence of the constitutively activated mutant.

Effects of anti-IgE and C5a on expression of CD63 on mast cells obtained from patients with ISM or ASM/MCL and from normal/reactive bone marrow

We found that 10 μ g/ml anti-IgE as well as 100 nM C5a upregulated the expression of CD63 on mast cells in normal bone marrow, whereas CD63 expression was not affected by anti-IgE or C5a in mast cells derived from patients with ISM or advanced SM (ASM/MCL) (Figure S7). These data corroborate those obtained *in vitro* with the ROSA^{KIT D816V} cells which were found unresponsive to IgE and non-IgE triggers (Figures S5A, S6B and S6D), as well with *ex vivo* data on BM mononuclear cells of SM patients, which failed to release histamine upon Fc ϵ RI triggering (Figure S5B and S5C).

Activated AKT and STAT5 are critical for the proliferation of ROSA^{KIT D816V} cells

The potential role of AKT and STAT5 in the proliferation of ROSA cells was evaluated by treating both cell lines with an AKT1/2 inhibitor (compound A6730; 0.01 to 5 μ M) or with a STAT5 inhibitor (pimozide; 1 to 6 μ M). Interestingly, as shown in Figure S8A, while the IC₅₀ of compound A6730 was

found to be 3 μM for ROSA^{KIT WT} cells, the same compound inhibited 50% of the proliferation of ROSA^{KIT D816V} cells already at 0.5 μM . Regarding pimozide, IC50 for ROSA^{KIT D816V} cells was found around 1 μM , whereas it was > 6 μM for ROSA^{KIT WT} cells (Figure S8B).

Engraftment of ROSA^{KIT D816V} cells and ROSA^{KIT WT} cells in NSG mice as determined by tryptase staining

Since we evidenced that intravenously injected ROSA^{KIT WT} cells and particularly ROSA^{KIT D816V} cells were able to engraft NSG mice in the bone marrow (Table 1), we sought to investigate for possible infiltration by the same cellular elements of other organs of the grafted NSG mice. For this purpose, tryptase staining by IHC was performed on sections prepared from paraffin-embedded, formalin-fixed BM, lung, liver and spleen of 5 NSG mice injected with ROSA^{KIT WT} cells and 5 NSG mice injected with ROSA^{KIT D816V} cells. Engraftment levels were defined using the following score: ++, infiltration grade >50%; +, infiltration 10-50%; +/-, 2-10%; -/+, only a very few cells detected; -, no engraftment found. Data presented in Table S4 showed that in NSG mice injected i.v. with ROSA^{KIT D816V} cells, the two main organs involved (apart BM) were spleen and lungs, with a more important involvement for the spleen (one mouse found ++, 3 found + and one found +/-/6) than for the lung (2 mice were found +, one mouse was found +/- and two mice were found negative). Interestingly, the liver seemed to be spared by ROSA^{KIT D816V} infiltration since 4 mice were found negative and only one mouse was scored +/- (this latter animal having the highest score concerning spleen involvement). Regarding NSG mice injected i.v. with ROSA^{KIT WT} cells, and in line with the lower level of BM involvement already observed for these animals, we observed a lower level of spleen involvement (score ranging from -/+ to +) and no lung or liver involvement, excepted for one mouse who presented with a low level (-/+) of

involvement for both organs (Table S4). All in all, these data confirmed the highest tumorigenic potential of ROSA^{KIT D816V} cells as compared to their KIT WT counterpart.

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Supplemental Tables

Table S1: Characteristics and origins of the various antibodies used in multicolor flow cytometry experiments.

Antigen	Clone	Fluorochrome	Isotype	Manufacturer
Isotype Control	MOPC-21	FITC	Mouse IgG1	BD Biosciences
Isotype Control	G155-178	FITC	Mouse IgG2a	BD Biosciences
Isotype Control	MOPC-21	PE	Mouse IgG1	BD Biosciences
Isotype Control	NA/LE	PE	Mouse IgG1	BD Biosciences
Isotype Control	polyclonal	PE	Mouse IgG1	R&D
Isotype Control	G155-178	PE	Mouse IgG2a	BD Biosciences
Isotype Control	133303	PE	Mouse IgG2b	R&D
Isotype Control	MOPC-21	APC	Mouse IgG1	BD Biosciences
CD2	RPA-2.10	PE	Mouse IgG1	BD Biosciences
CD3	UCHT1	APC	Mouse IgG1	BD Biosciences
CD4	RPA-T4	FITC	Mouse IgG1	BD Biosciences
CD5	L17F12	FITC	Mouse IgG2a	BD Biosciences
CD8	RPA-T8	PE	Mouse IgG1	BD Biosciences
CD9	ALB 6	FITC	Mouse IgG1	Beckman Coulter
CD10	HI10a	PE	Mouse IgG1	BD Biosciences
CD11b	ICRF44	PE	Mouse IgG1	Biolegend
CD13	WM15	PE	Mouse IgG1	BD Biosciences
CD14	TÜK4	FITC	Mouse IgG2a	Dako
CD15	W6D3	PE	Mouse IgG1	Biolegend

CD16	NKP15	FITC	Mouse IgG1	BD Biosciences
CD17	G035	FITC	Mouse IgG1	BD Biosciences
CD19	4G7	FITC	Mouse IgG1	BD Biosciences
CD20	L27	PE	Mouse IgG1	BD Biosciences
CD22	SJ10.1H11	PE	Mouse IgG1	Beckman Coulter
CD23	M-L233	PE	Mouse IgG1	BD Biosciences
CD24	ML5	FITC	Mouse IgG2a	BD Biosciences
CD25	2A3	PE	Mouse IgG1	BD Biosciences
CD26	M-A261	PE	Mouse IgG1	BD Biosciences
CD27	O323	PE	Mouse IgG1	Biolegend
CD30	BerH8	PE	Mouse IgG1	BD Biosciences
CD31	AC128	APC	Mouse IgG1	Miltenyi Biotec
CD32	2E1	PE	Mouse IgG2a	Beckman Coulter
CD33	WM53	PE	Mouse IgG1	BD Biosciences
CD34	581	PE	Mouse IgG1	BD Biosciences
CD38	HIT2	APC	Mouse IgG1	BD Biosciences
CD44	515	PE	Mouse IgG1	BD Biosciences
CD45	2D1	PerCP	Mouse IgG1	BD Biosciences
CD45	2D1	APC	Mouse IgG1	BD Biosciences
CD48	J4-57	FITC	Mouse IgG1	Beckman Coulter
CD50	ICAM3.3 (CH3.3)	FITC	Mouse IgG1	Serotec
CD52	HI186	PE	Mouse IgG2b	R&D
CD54	84H10	FITC	Mouse IgG1	Beckman Coulter

CD56	NCAM16.2	PE	Mouse IgG1	BD Biosciences
CD58	AICD58	FITC	Mouse IgG2a	Beckman Coulter
CD59	p282 (H19)	FITC	Mouse IgG2a	BD Biosciences
CD63	CLBGran/12	FITC	Mouse IgG1	Beckman Coulter
CD64	10,1	FITC	Mouse IgG1	BD Biosciences
CD66a	283340	PE	Mouse IgG2b	R&D
CD69	FN50	FITC	Mouse IgG1	BD Biosciences
CD71	MEM-75	FITC	Mouse IgG1	Biolegend
CD87	VIM5	PE	Mouse IgG1	Biolegend
CD90	5E10	PE	Mouse IgG1	BD Biosciences
CD95	DX2	PE	Mouse IgG1	BD Biosciences
CD96	NK92.39	PE	Mouse IgG1	eBiosciences
CD105	166707	PE	Mouse IgG1	R&D
CD114	LMM741	PE	Mouse IgG1	BD Biosciences
CD115	61708	PE	Mouse IgG1	R&D
CD116	31916	PE	Mouse IgG1	R&D
CD117	104D2	PE	Mouse IgG1	BD Biosciences
CD123	32703	PE	Mouse IgG1	R&D
CD127	hIL-/R-M21	PE	Mouse IgG1	BD Biosciences
CD133	AC133	PE	Mouse IgG1	Miltenyi Biotec
CD134	ACT35	PE	Mouse IgG1	BD Biosciences
CD138	DL-101	PE	Mouse IgG1	BD Biosciences
CD144	123413	PE	Mouse IgG2b	R&D

CD146	128018	PE	Mouse IgG1	R&D
CD150	A12 (7D4)	PE	Mouse IgG1	Biolegend
CD164	N6B6	PE	Mouse IgG2a	BD Biosciences
CD166	105902	PE	Mouse IgG1	R&D
CD203c	97A6	PE	Mouse IgG1	Beckman Coulter
CD213a	B-K19	PE	Mouse IgG1	cell sciences
CD218a	H44	PE	Mouse IgG1	Biolegend
CD271	C40-1457	PE	Mouse IgG1	BD Biosciences
CD273	MIH18	PE	Mouse IgG1	Biolegend
CD274	MIH1	PE	Mouse IgG1	BD Biosciences
CD279	EH12.2H7	PE	Mouse IgG1	Biolegend
CD300a	MEM-260	PE	Mouse IgG1	abcam
CD309	89106	PE	Mouse IgG1	R&D

Table S2: Analysis by flow cytometry of several surface markers expressed by ROSA cell lines. The expression of selected cell surface antigens was analyzed by direct immunofluorescence using FACSCalibur and Aria flow cytometer after cell labeling by specific monoclonal anti-human antibodies directed against the specified CD. Intensity of the labeling for each antigen is expressed as Mean Fluorescence Intensity ratio (MFI test mAb/MFI control mAb) MFI ratio < 1.19:-, MFI ratio 1.2 - 2.9:+/-, MFI ratio 3-9.9: +, MFI ratio 10-99.9: ++, MFI ratio >100:+++ . N.t.: not tested.

	ROSA^{KIT WT}	ROSA^{KIT D816V}		ROSA^{KIT WT}	ROSA^{KIT D816V}
CD2	-	-	CD59	+/-	n.t.
CD3	-	-	CD63	+	++
CD4	++	n.t.	CD64	+/-	n.t.
CD5	-	n.t.	CD66a	+++	++
CD8	-	-	CD69	+	n.t.
CD9	++	n.t.	CD71	+	n.t.
CD10	-	-	CD87	+	++
CD11b	+/-	+/-	CD90	-	+
CD13	+/-	++	CD95	-	-
CD14	-	-	CD96	+/-	-
CD16	-	n.t.	CD105	-	-
CD17	-	n.t.	CD114	-	-
CD19	-	-	CD115	-	n.t.
CD20	-	-	CD116	-	-
CD22	+	-	CD117	++	+++
CD23	-	-	CD123	+/-	+/-
CD24	+/-	n.t.	CD127	+/-	-
CD25	-	-	CD133	-	-
CD30	-	-	CD134	+/-	-
CD31	+	-	CD138	-	-
CD32	++	++	CD144	+/-	-
CD33	++	++	CD146	+/-	-
CD34	-	-	CD150	+/-	+/-
CD38	-	-	CD164	+	+/-
CD44	+++	+++	CD166	++	++
CD45	+++	+++	CD203c	++	++
CD48	+	n.t.	CD213a	-	-
CD50	++	n.t.	CD218a	+/-	-
CD52	++	++	CD279	-	-
CD54	+	n.t.	CD300a	++	++
CD56	-	+	CD309	+/-	-

Table S3: Major demographic and biological characteristics of mastocytosis patients involved in the study.

Pat. No	m/f	Age	Diagnosis (WHO)	Tryptase level (ng/ml)	<i>KIT</i> codon 816 mutation	% MC in BM sections	% MC in BM smears	% MC in PB smears
1	m	71	ASM	88.6	D816V+	15%	1%	0%
2	f	57	ISM	30.7	D816V+	5%	<1%	0%
3	m	64	ISM	33.5	D816V+	5%	3%	0%
4	f	54	MCL	904	D816H+	50%	60%	75%
5	f	68	ISM-MGUS	41	n.t.	<5%	1%	0%
6	m	75	ASM	75.5	D816V+	10%	<1%	0%
7	f	74	SM-CMML	67.5	D816V+	5%	1%	0%
8	f	48	MCL	332	D816V-	60%	70%	0%
9	m	68	ASM	152	D816V+	5-10%	2%	0%
10	m	48	ISM	40.9	D816V+	10%	1%	0%
11	m	48	ISM	82.5	D816V+	5%	1%	0%
12	f	48	ISM	34.1	D816V+	5%	1%	0%

m, male; f, female; ASM, aggressive systemic mastocytosis; ISM, indolent systemic mastocytosis; MCL, mast cell leukemia; MGUS, monoclonal gammopathy of undetermined significance; SM-CMML, SM with an associated Chronic Myelomonocytic Leukemia. MC: mast cell; BM: bone marrow; PB: peripheral blood; WHO: World Health Organization.

*Serum tryptase levels were determined at diagnosis by commercial fluoroimmunoassay (FIA; Pharmacia, Uppsala, Sweden). Normal value for tryptase level in serum: < 15 ng/mL.

Table S4: Engraftment of ROSA^{KIT D816V} cells and ROSA^{KIT WT} cells in NSG mice as determined by tryptase staining. Tissue sections obtained from NSG mice injected with ROSA^{KIT D816V} cells and ROSA^{KIT WT} cells, were stained with an antibody against mast cell tryptase. Engraftment levels were defined using the following score: ++, infiltration grade >50%; +, infiltration 10-50%; +/-, 2-10%; -/+, only a very few cells detected; -, no engraftment found. Abbreviations: WT, wild type.

Mouse number	Bone marrow	Liver	Lung	Spleen
186/D816V	++	+/-	+	++
187/D816V	++	+/-	+/-	+
190/D816V	++	-	+/-	+
191/D816V	++	-	+	+
171/WT	++	+/-	+/-	+
172/WT	-/+	-	-	-/+
173/WT	+	-	-	+
174/WT	++	-	-	+
175/WT	+/-	-	-	-/+

Table S5: Level of chymase expression in ROSA^{KIT D816V} cells engrafting in NSG mice, as determined by immunohistochemistry (IHC) on biopsies of sub-cutaneous tumors or of infiltrated bone marrow (BM) samples. NSG mice were injected i.v. or s.c. with ROSA^{KIT D816V} cells as described in the Material and Methods section. The diseased mice were sacrificed and BM cells were obtained from flushed femurs, tibias, and humeri, whereas tumors grown from s.c. injected cells were collected and dispersed by collagenase digestion. The percentage of CD117 positive cells was determined for each sample by flow cytometry. IHC for chymase was performed on slides prepared from paraffin-embedded, formalin-fixed BM and tumor biopsy specimens. The percentage of chymase positive cells was determined using a scoring system.

No.	ROSA Injected	Mouse No.	Flow Cytometry	IHC
			CD117 (%)	Chymase
P751 Tumor	D816V	30	58	+
P752 Tumor	D816V	30	58	+/-
P757 BM	D816V	186	66	-
P761 BM	D816V	187	76	-

Score Result: +, most cells immunoreactive; +/-, 10-50% of cells reactive; -, no chymase+ cells found.

Supplemental Figures

Supplemental Figure S1

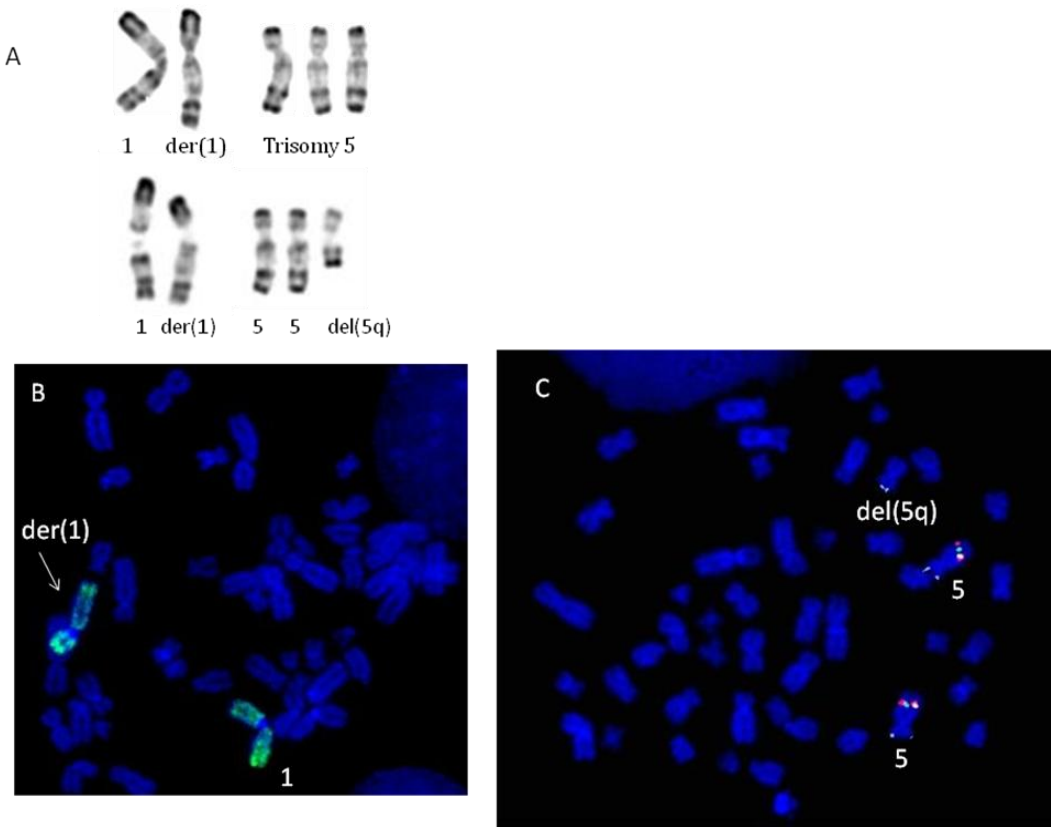


Figure S1: Partial karyotype and Fluorescent *in situ* Hybridization (FISH) analyses of ROSA^{KIT} WT cells after 12 months of culture. A: Partial R-banding karyotype. Upper panel: sub-clone with a derived chromosome 1 and a complete trisomy 5. Lower panel: main sub-clone with the derived chromosome 1 and a partial trisomy 5. The complete karyotype was: 46,XY, der(1)inv(1)(p31q21)del(1)(q24q32)[4]47,sl,+5[3]/47,sl,+del(5)(q14q34)[13]. B and C: FISH analyses. B. Representative metaphase hybridized with the Whole Chromosome Painting (WCP1) probe, labeled in green. The inverted/deleted derived chromosome 1 is entirely hybridized with the WCP1 probe. B. Representative metaphase hybridized with the EGR1(5q31) (green), CSF1R(5q33) (red) and hTERT(5p15) (blue) probes (Kreatech) Confirmation of the partial trisomy 5 with the presence of two normal chromosomes 5 hybridized with the three probes, and one additional deleted chromosome 5, del(5q), with loss of EGR1 and CSF1R probes while the hTERT probe on the short arm remained. This profile was observed in 10/15 metaphases analyzed.

Supplemental Figure S2

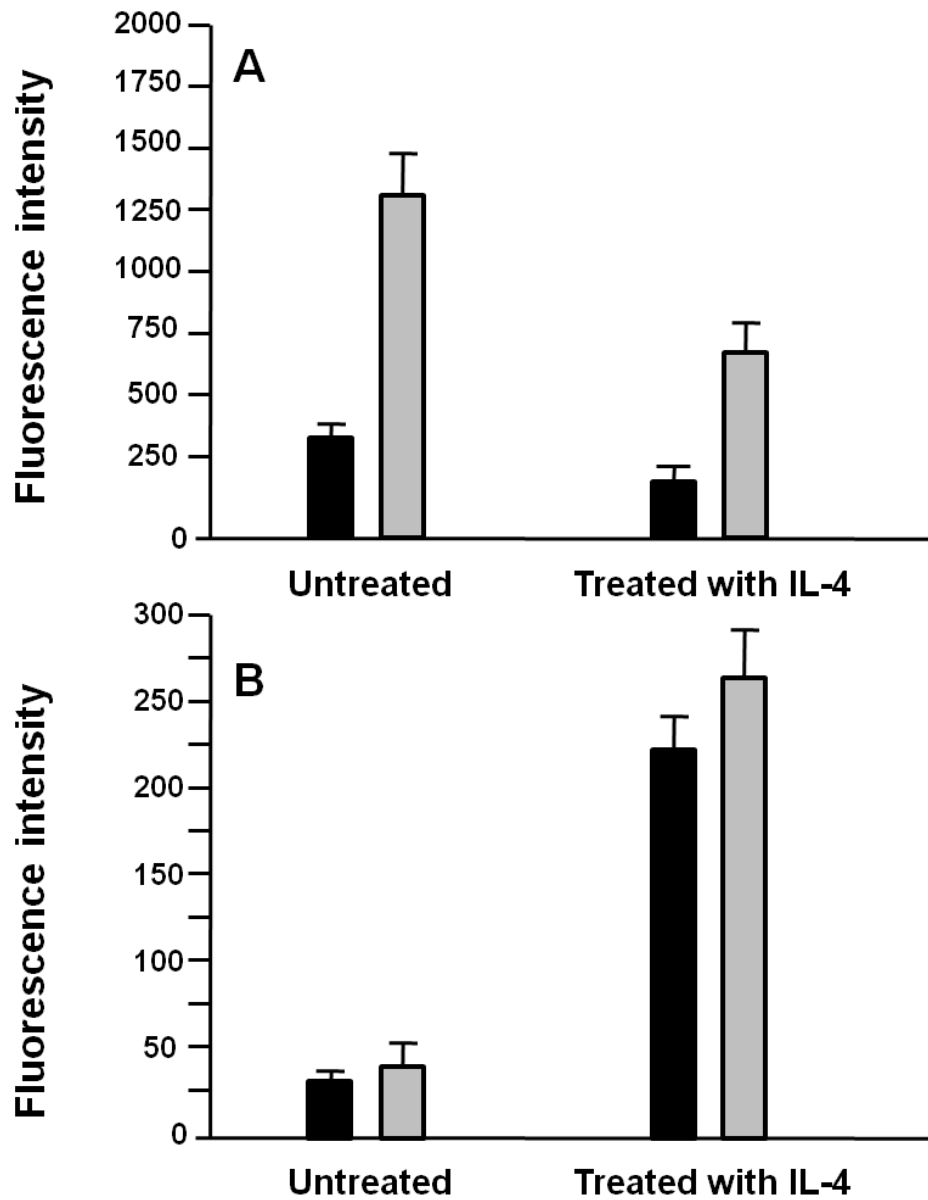


Figure S2: Effect of IL-4 on the expression of KIT (A) and FcεRI (B) by the two different ROSA cell lines. ROSA^{KIT^{WT}} (black bars) or ROSA^{KIT^{D816V}} (grey bars) cells were treated with IL-4 (20 ng/ml) for 5 days, washed, then incubated in the presence of anti-human KIT (CD117) or with anti-human FcεRI, both coupled with APC. After labeling, the fluorescence intensity was measured using a FACSCalibur. Data presented are the mean ± SD from 3 independent experiments.

Supplemental Figure S3

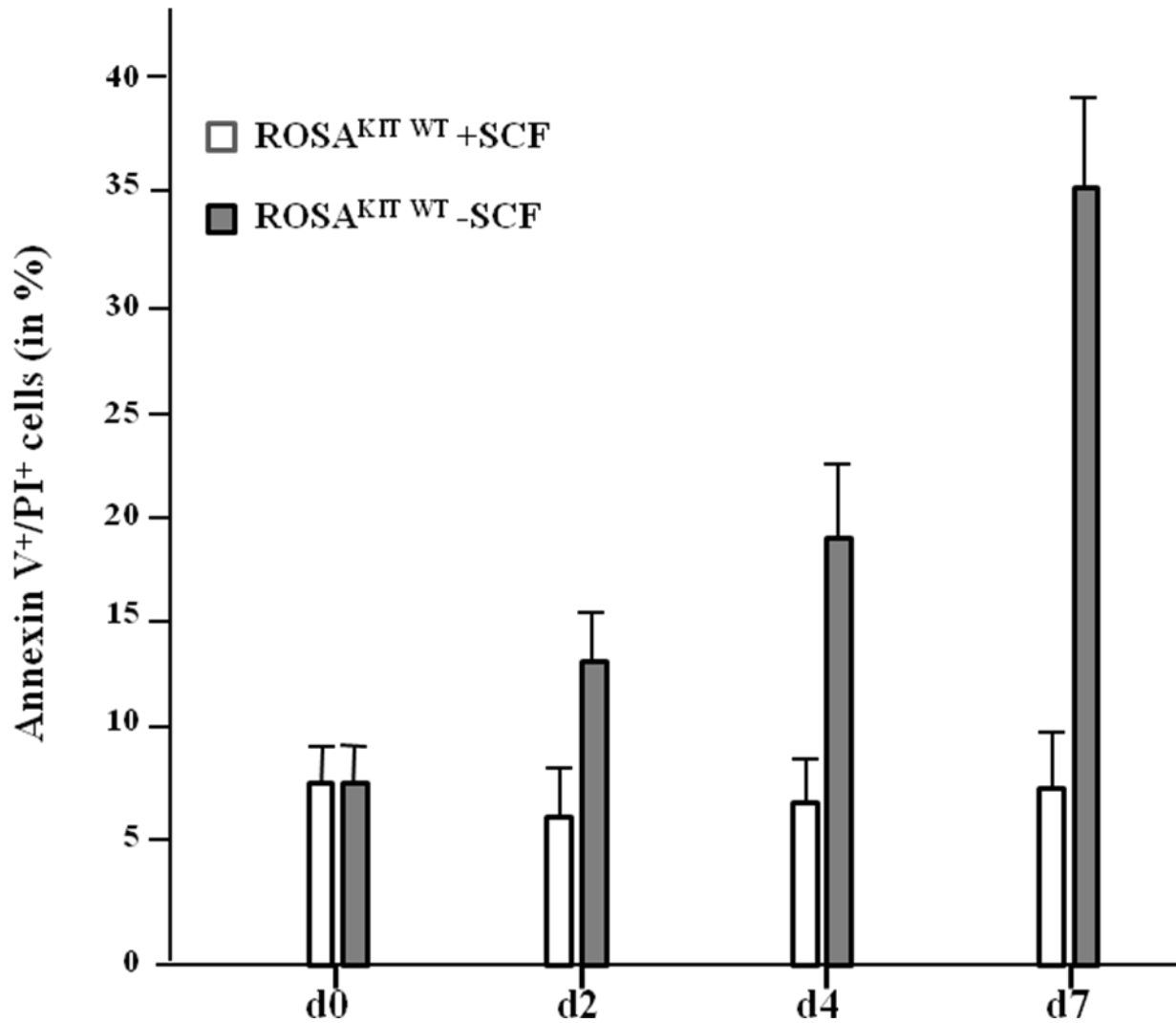


Figure S3: Effect of withdrawal of SCF on the apoptosis of ROSA^{KIT WT} cells. ROSA^{KIT WT} were treated or not with recombinant human SCF (80 ng/ml), then cultured for 7 days in an incubator at 37°C (5% CO₂). At the beginning of the culture (d0) and at each time point (d2, d4 and d7), an aliquot of the cells (10⁶) in both conditions was labeled with Annexin V-APC/propidium iodide (PI). The percentage of Annexin V positive/PI positive cells was then measured for each condition by flow cytometry with a FACSCalibur flow cytometer. Data presented are the mean ± SD from 3 independent experiments.

Supplemental Figure S4

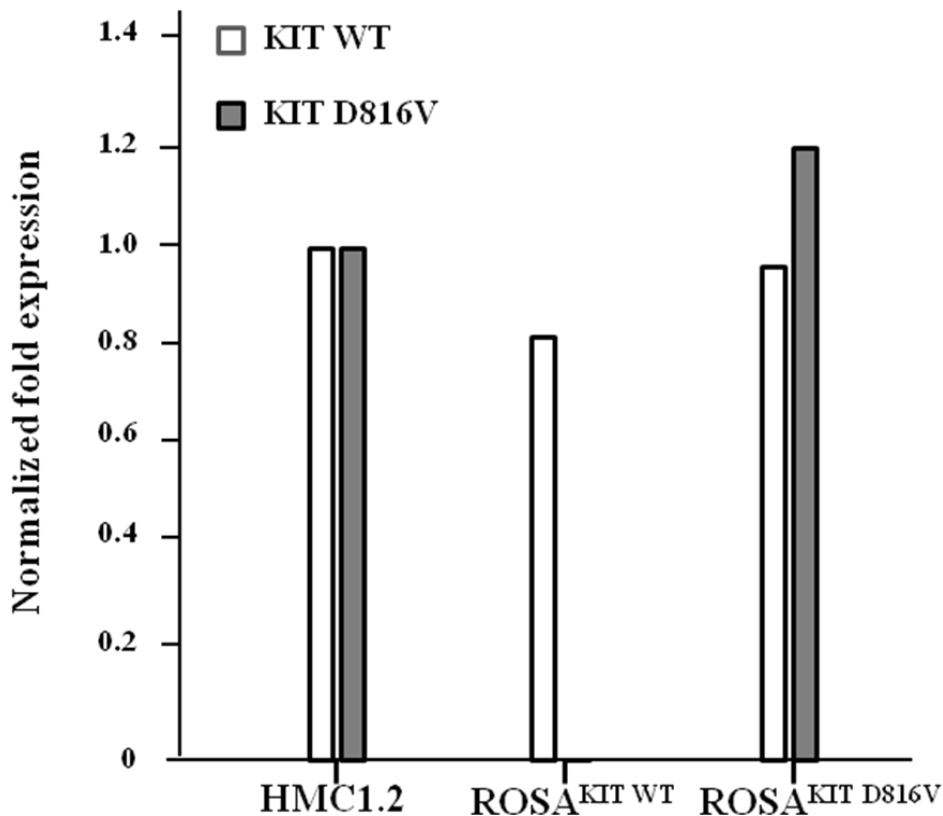


Figure S4: Quantitative expression analysis of mRNA coding for KIT WT or for KIT D816V in ROSA^{KIT WT} or ROSA^{KIT D816V} cells as compared to HMC1.2 cells using qPCR with specific primers. Total RNA from 5.10⁶ ROSA^{KIT WT} and ROSA^{KIT D816V} cells, from 5.10⁶ HMC1.2 cells used as the control, was isolated then reverse transcribed into cDNA. Real time PCR was then using primers specific for KIT WT or for KIT D816V cDNA. Reactions were compared to the levels of expression of mRNA for Hypoxanthine Phosphoribotransferase (HPRT). A serial dilution of the cDNA mixture was used to produce a standard curve and the cDNA of HMC1.2 was used as a calibrator for gene expression analysis. The mRNA expression of KIT WT and KIT D816V in the two ROSA cell lines was calculated relative to the expression of these genes in control HMC1.2, according to the delta-delta C_t method (2^{-ΔΔC_t}). Data are reported as normalized fold expression of each mRNA using the 2^{-ΔΔC_t}

Supplemental Figure S5

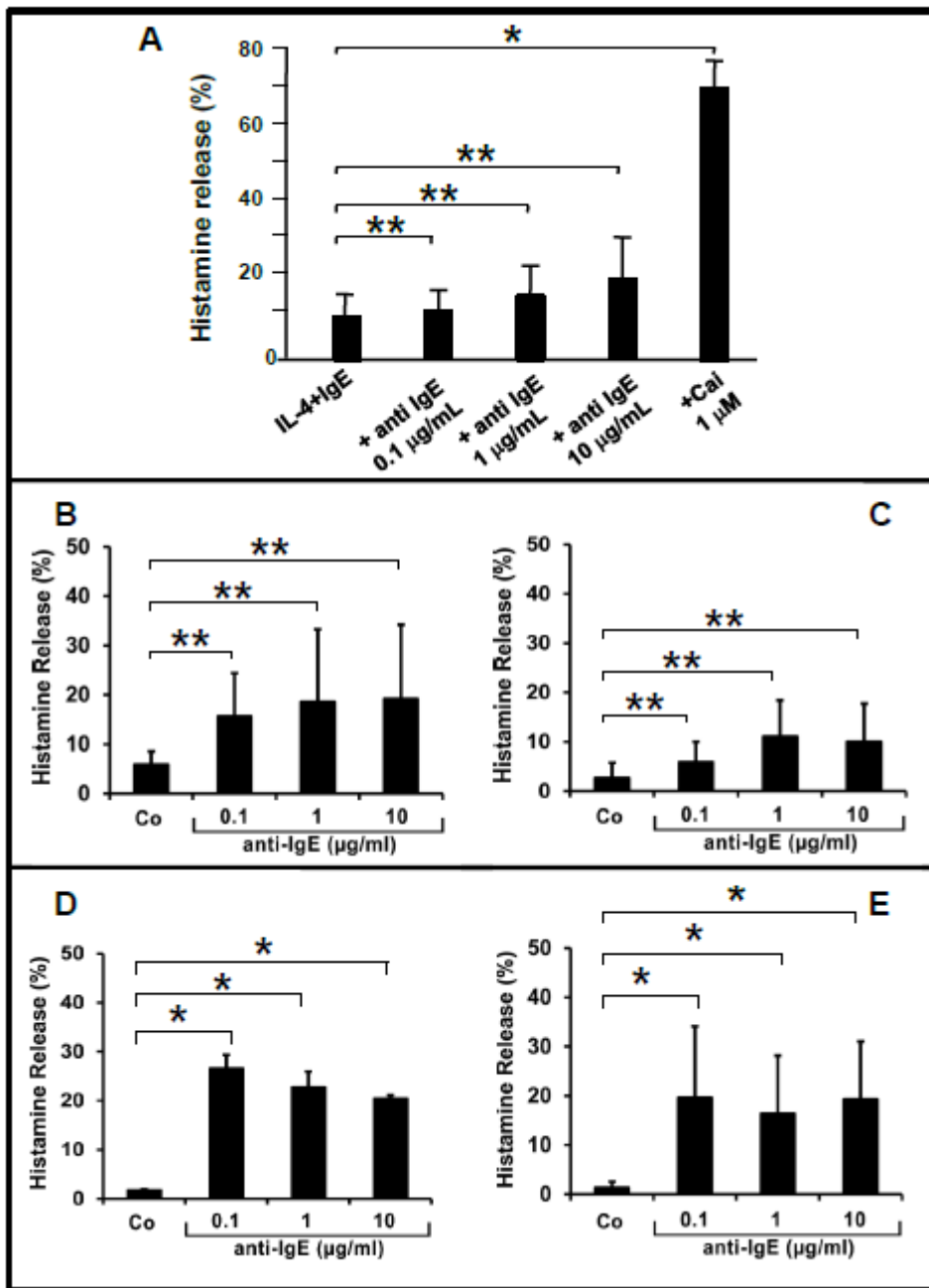


Figure S5: Percentage of histamine released from ROSA^{KIT D816V} (A), from BM cells from mastocytosis patients (B and C), from PB dextran-enriched mononuclear cells isolated from mastocytosis patients (D) or from PB dextran-enriched basophils isolated from 3 normal donors.

A: primed ROSA^{KIT D816V} cells were stimulated with various concentrations of anti-human anti-IgE antibody at 37°C for 30 min. Thereafter, cells were centrifuged at 4°C, and the supernatants and cell pellets were analyzed for histamine content using a specific radioimmunoassay kit. Results of histamine release are expressed as percent of total histamine and represent the mean ± SD of three independent experiments. **B and C:** percentage of histamine release from BM derived mononuclear

cells isolated from 7 patients with mastocytosis after preincubation with human recombinant SCF. Bone marrow (BM) derived primary mononuclear cells (MNC) obtained from 3 patients with indolent systemic mastocytosis (ISM) (patients N° 2, 3 and 5 of the Table S3), from 2 patients with aggressive systemic mastocytosis (ASM) (patients N° 1 and 6 of the Table S3) and from 2 patients with mast cell leukemia (MCL) (patients N° 4 and 8 of the Table S3) were incubated with 200 ng/ml human recombinant SCF at 37°C for 90 minutes. Then, cells were incubated with three concentrations of anti-IgE for 30 minutes. Thereafter, histamine was quantified as described above in the supplemental methods section. **A:** mean \pm SD of the percent of histamine release for the ISM patients; **B:** mean \pm SD of the percent of histamine release for 2 ASM and 2 MCL patients. Results represent the mean \pm SD of 7 independent experiments. **D:** percentage of histamine release from PB dextran-enriched mononuclear cells isolated from 2 patients with ISM and 2 others with ASM. **E:** Percentage of histamine release from PB dextran-enriched basophils isolated from 3 normal donors. PB derived primary mononuclear cells (MNC) obtained from 4 patients with systemic mastocytosis (2 ISM and 2 ASM), as well as Dextran-enriched basophils from 3 healthy donors, were preincubated in medium containing 200 ng/ml human recombinant SCF at 37°C for 90 minutes. Then, cells were incubated with three concentrations of anti-IgE for 30 minutes. Thereafter, histamine was quantified in cell lysates and supernatants by RIA. Histamine release is expressed as the percentage of total histamine and results represent the mean \pm SD of four independent experiments. Data were analyzed by the Student's t-test and differences were considered to be statistically significant when p was <0.05 . Asterisk (*): $p < 0.05$, asterisk (**): $p > 0.05$.

Supplemental Figure S6

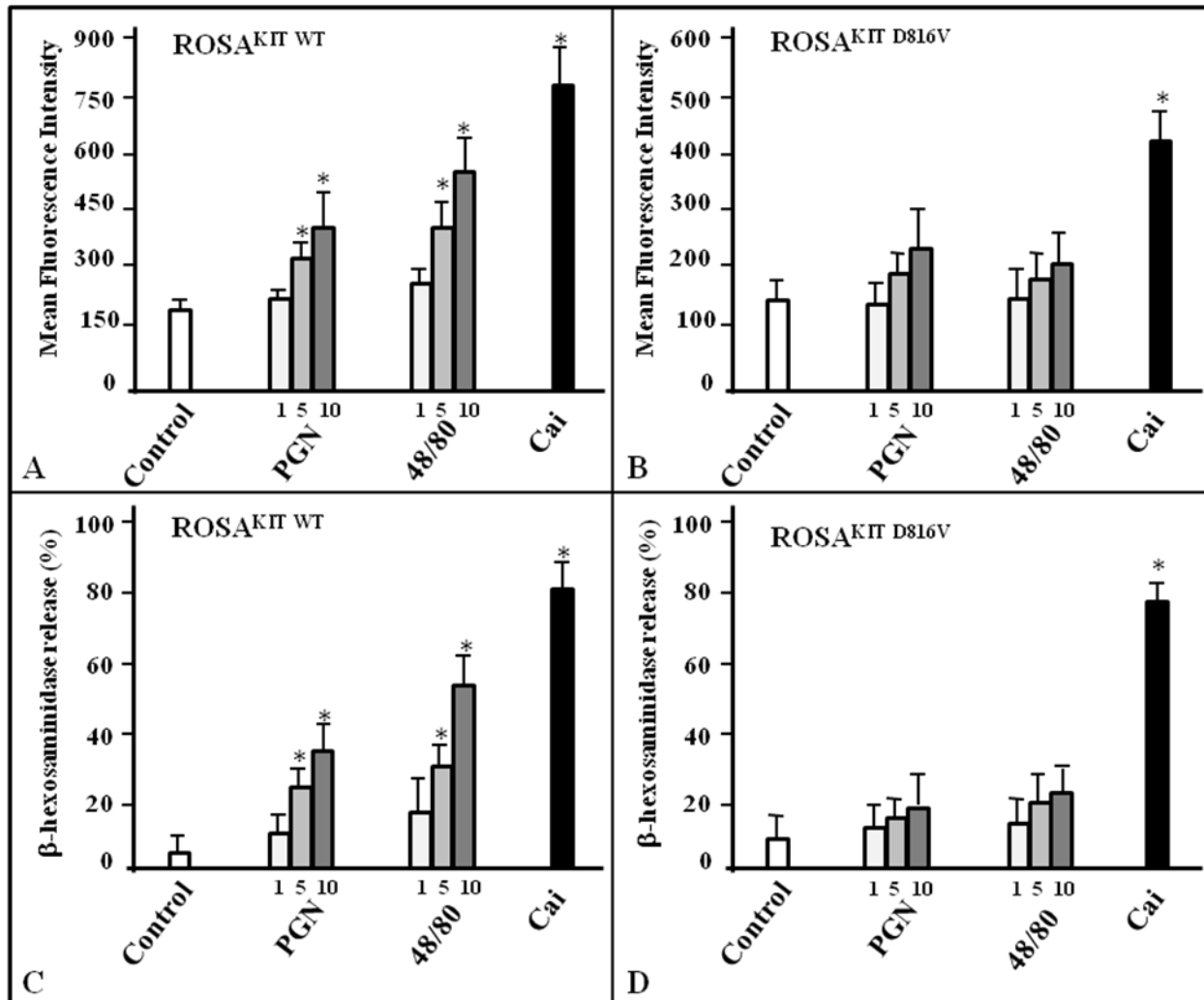


Figure S6: Biological effects of non-IgE triggers on ROSA cell lines. A and B: *CD203c* up-regulation: ROSA^{KIT WT} (A) or ROSA^{KIT D816V} (B) cells were stimulated or not by peptidoglycan (PGN) from *S. aureus* (PGN) (1, 5 or μ g/mL), by compound 48/80 (1, 5 or μ g/mL) by Ca-ionophore (1 μ M) (Cai) for 1 hour. After labeling with an anti-human CD203c Ab coupled with APC, the fluorescence intensity was measured using a FACSCalibur. C and D: β -hexosaminidase release: ROSA^{KIT WT} (C) or ROSA^{KIT D816V} (D) cells were stimulated or not by peptidoglycan (PGN) from *S. aureus* (PGN) (1, 5 or μ g/mL), by compound 48/80 (1, 5 or μ g/mL) by Calcium ionophore (1 μ M) (Cai) for 1 hour. β -hexosaminidase enzymatic activity was measured in the cell-free supernatants and after cell lysis as described in the methods section. For each parameter, data presented are the mean \pm SD from 3 independent experiments. *: value significantly different from the control at $p < 0.05$.

Supplemental Figure S7

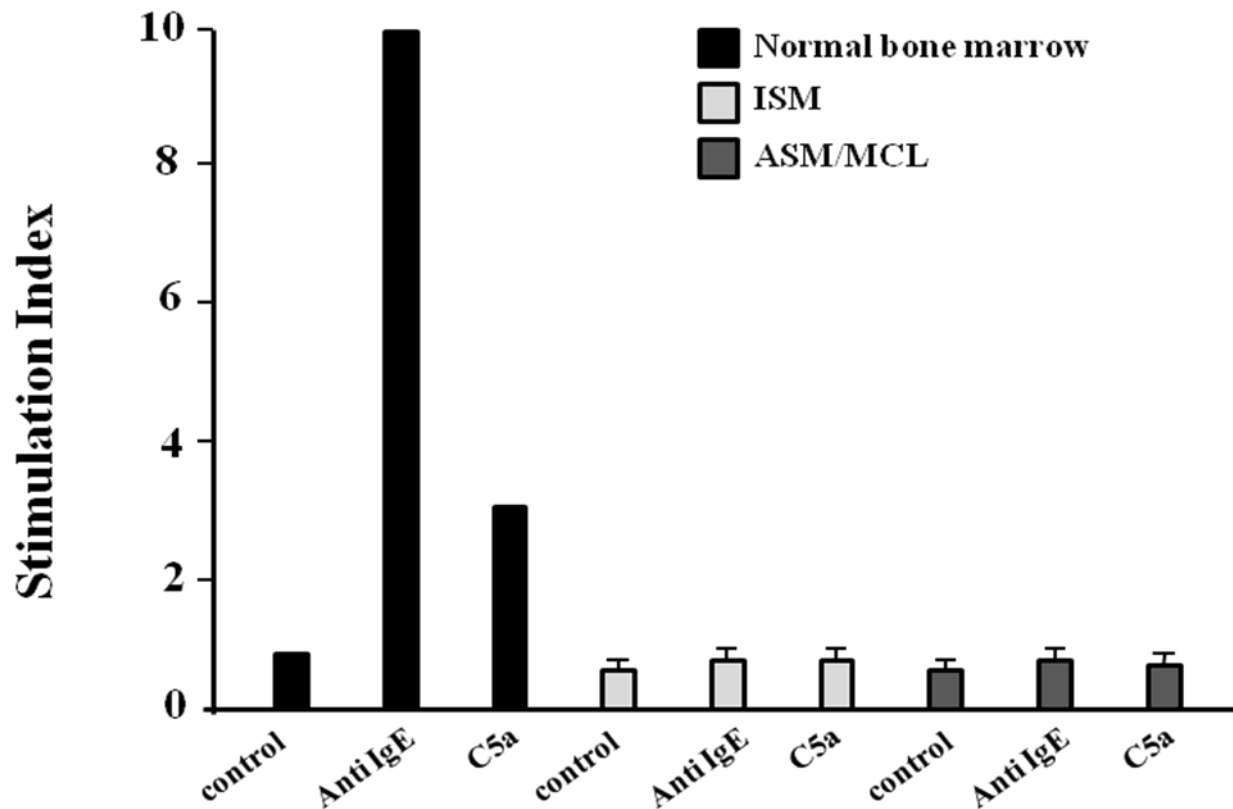


Figure S7: Effects of anti-IgE and C5a on the expression of CD63 on mast cells obtained from patients with ISM or ASM/MCL and from normal/reactive bone marrow. Unseparated BM cells from *KIT* D816X+ SM patients (ISM, n=3, Patients N° 10, 11 and 12 of the Table S3; ASM/MCL, n=3, Patients N° 1, 4 and 9 of the Table S3) and from normal/reactive bone marrow (n=1) were incubated with anti-IgE mAb (10 µg/ml), recombinant human C5a (100 nM) or control buffer at 37°C for 90 minutes. Thereafter, cells were washed and stained with FITC-labeled CD63 mAb, PE-Cy-7-labeled CD117 mAb 104D2, APC-Cy7-labeled CD45 mAb, and Pacific Blue-labeled CD34 mAb 581 for 15 minutes at room temperature. After erythrocyte lysis, expression of cell surface antigens was quantified by multi-color flow cytometry. MCs were identified as CD45+/CD117+/CD34- cells. Anti-IgE-induced and C5a-induced upregulation of CD63 on MCs was calculated from mean fluorescence intensities (MFI) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells and was expressed as stimulation index = SI (MFI_{stim} : MFI_{control}). In the ISM- and ASM/MCL samples, results represent the mean±S.D. from three independent experiments (3 donors each).

Supplemental Figure S8

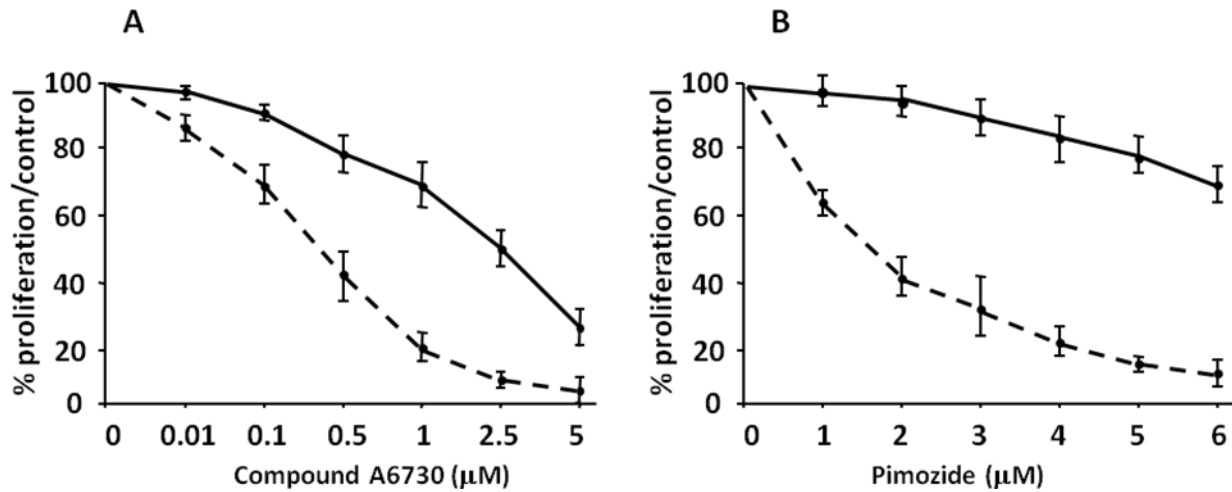


Figure S8: Effect of the specific inhibition of AKT (A) and STAT5 (B) on the proliferation of the two ROSA cell lines. ROSA^{KIT^{WT}} (—) or ROSA^{KIT^{D816V}} (---) cells were seeded for 48 hours in the presence of various concentrations (0.01 to 5 μM) of the selective inhibitor of AKT1/AKT2 (compound A6730 diluted in DMSO 0.1% final concentration) or in the presence of various concentrations (1 to 6 μM) of the specific STAT5 inhibitor (pimozide diluted in DMSO 0.1% final concentration) in SFM (with rhSCF at 80 ng/mL for ROSA^{KIT^{WT}} but not for ROSA^{KIT^{D816V}} cells). At the end of this incubation, 10 μL of MTT were added in each well (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and the cells were incubated for 3 additional hours in an incubator at 37°C. The number of living cells was then measured for each condition by reading the absorbance at 570 nm. Data presented are the mean \pm SD from 3 independent experiments and are expressed as percent of proliferation in each condition relative to the control (DMSO alone) considered as 100% proliferation.

Supplemental Figure S9

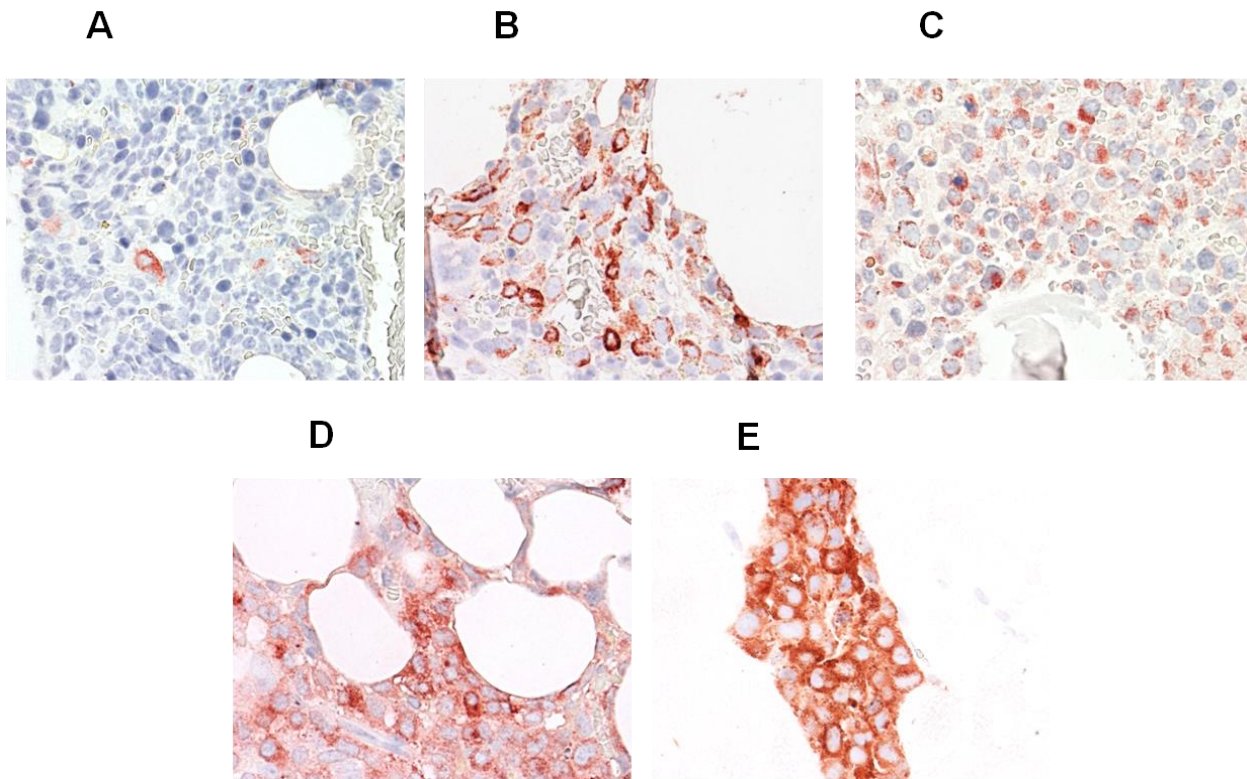


Figure S9: Engraftment of ROSA^{KIT D816V} cells in NSG mice. Correlation between the percentage of human CD45⁺/CD117⁺ cells measured by Flow Cytometry (FACS) on BM cells in suspension and intensity of tryptase staining on BM biopsy of the same animal. A: 7% of human MCs by FACS; B: 37% of human MCs by FACS; C: 66% of human MCs by FACS; D: 76% of human MCs by FACS; E: Local MC Tumor with nearly 100% of human MCs by FACS. For tryptase staining, IHC was performed with the anti-Tryptase mAb G3 as described in the supplemental methods section. Magnification is x 100.

RESULTS

Publication 2. "Identification of bromodomain-containing protein-4 as a novel marker and epigenetic target in mast cell leukemia"

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RESULTS

Publication 2. "Identification of bromodomain-containing protein-4 as a novel marker and epigenetic target in mast cell leukemia"

In this second article, we reported a role for BRD4 as potential target in neoplastic MCs in advanced SM (Wedeh *et al.* 2015). Indeed, we found that BM-derived neoplastic MCs from patients with ASM and MCL expressed substantial amounts of cytoplasmic BRD4 compared to MCs in patients with ISM. In addition, we observed that the human MC lines HMC-1.1, HMC-1.2, ROSA^{KIT WT} and ROSA^{KIT D816V} expressed cytoplasmic and nuclear BRD4. Moreover, the shRNA-induced knock-down of BRD4 resulted in a significant growth inhibition of HMC-1 cells and ROSA cells, and induced apoptosis in these cells. Also, we found that JQ1, a specific inhibitor of BRD4, induced a cell cycle arrest in G1 in these cells. Furthermore, JQ1 down-regulated dose-dependently the expression of CD63 and CD71 in HMC-1 cells and ROSA cells. In addition, we have observed that JQ1 downregulated the expression of FcεRIα in ROSA^{KIT WT} cells. Consistent with this latter data, we found that JQ1 blocked IgE-mediated histamine release and counteracted IgE-mediated up-regulation of CD63 and CD203c in ROSA^{KIT WT} cells. Besides, the growth-inhibitory effects of JQ1 on neoplastic human MC lines were associated with down-regulation of the expression of BCL-2, MCL-1, BCL-xL and MYC mRNA, and up-regulation of the expression of BIM mRNA.

We next examined the mechanisms underlying the expression of BRD4 in neoplastic MCs in SM. We found that SCF upregulated BRD4 mRNA expression in ROSA^{KIT D816V} cells but not in HMC-1 cells.

To explore underlying signalling pathways, we applied KIT-targeting drugs and a panel of signal transduction inhibitors. Interestingly, the KIT-targeting drugs PKC412 and dasatinib suppressed the expression of BRD4 mRNA in all human MC lines tested. Besides, Imatinib was found to suppress the expression of BRD4 mRNA in HMC-1.1 cells and ROSA^{KIT WT} cells, but was not able to block BRD4 expression in HMC-1.2 or ROSA^{KIT D816V} cells. In addition, we observed that the MEK inhibitors RDEA119 and PD0325901, the PI3K/mTOR blocker BEZ235 and all-trans retinoic acid (ATRA) were capable to downregulate the expression of BRD4 mRNA in HMC-1 cells and ROSA cells.

In the last part of our work, we screened for suitable anti-neoplastic drugs that might be applied in

combination with JQ1 in patients with advanced SM. We found that JQ1 strongly synergized with PKC412 and with ATRA in suppression of the growth of HMC-1 cells and ROSA cells and in induction of apoptosis. Interestingly, we observed that the combination 'JQ1+PKC412' was able to overcome the SCF-induced resistance in ROSA^{KIT D816V} cells.

ORIGINAL ARTICLE

Identification of bromodomain-containing protein-4 as a novel marker and epigenetic target in mast cell leukemia

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Advanced systemic mastocytosis (SM) is a life-threatening neoplasm characterized by uncontrolled growth and accumulation of neoplastic mast cells (MCs) in various organs and a poor survival. So far, no curative treatment concept has been developed for these patients. We identified the epigenetic reader bromodomain-containing protein-4 (BRD4) as novel drug target in aggressive SM (ASM) and MC leukemia (MCL). As assessed by immunohistochemistry and PCR, neoplastic MCs expressed substantial amounts of BRD4 in ASM and MCL. The human MCL lines HMC-1 and ROSA also expressed BRD4, and their proliferation was blocked by a BRD4-specific short hairpin RNA. Correspondingly, the BRD4-targeting drug JQ1 induced dose-dependent growth inhibition and apoptosis in HMC-1 and ROSA cells, regardless of the presence or absence of KIT D816V. In addition, JQ1 suppressed the proliferation of primary neoplastic MCs obtained from patients with ASM or MCL (IC50: 100–500 nM). In drug combination experiments, midostaurin (PKC412) and all-*trans* retinoic acid were found to cooperate with JQ1 in producing synergistic effects on survival in HMC-1 and ROSA cells. Taken together, we have identified BRD4 as a promising drug target in advanced SM. Whether JQ1 or other BET-bromodomain inhibitors are effective *in vivo* in patients with advanced SM remains to be elucidated.

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INTRODUCTION

Systemic mastocytosis (SM) is a hematopoietic stem cell disorder characterized by pathologic expansion and accumulation of clonal mast cells (MCs) in one or more internal organs.^{1–5} Indolent and advanced types of the disease have been described.^{1–5} The classification of the World Health Organization (WHO) delineates four major categories of SM, namely indolent SM (ISM), SM with an associated clonal hematopoietic non-MC-lineage disease (SM-AHNMD), aggressive SM (ASM) and MC leukemia (MCL).^{6–8} The smoldering subtype of SM, initially considered a subtype of ISM, is now also regarded a separate SM category.⁹ In ISM, the prognosis is quite favorable. In fact, despite mediator-related symptoms, cosmetic problems and the neoplastic nature of the disease, patients with ISM have a normal or near-normal life expectancy.^{5–8} By contrast, in patients with advanced SM (ASM, SM-AHNMD, MCL) the prognosis is grave.^{6–8,10–15} In these patients, cytoreductive therapy is administered to suppress MC expansion in various organ systems.^{9,10,12–16} However, unfortunately, clonal MCs in these patients are often resistant against anticancer drugs. Therefore, research efforts focus on new potential drug targets in neoplastic MCs and the development of novel targeted drugs for advanced SM.^{16–18}

One potential drug target in ASM and MCL is KIT D816V, an SM-related oncoprotein.^{17,18} This transforming mutant is detectable in a majority of all patients with SM, including ASM and MCL.^{19–21} KIT D816V is expressed in MCs as well as in MC progenitors in

these patients, and is considered to contribute substantially to differentiation and survival of neoplastic MCs.^{21–23} However, so far, only a few tyrosine kinase inhibitors were found to counteract the growth and survival of KIT D816V+ neoplastic MCs in patients with advanced SM.^{18,24–27} One of the most encouraging agents is the multikinase inhibitor PKC412, also known as midostaurin. However, despite impressive efficacy, responses to PKC412 in ASM and MCL are often incomplete or short lived.^{27,28} Therefore, research is seeking additional drug targets expressed by neoplastic MCs.

During the past few years, chromatin-regulatory molecules and related antigens have been examined as potential therapeutic targets in diverse human malignancies.^{29–31} One promising class of targets are the bromodomain-containing proteins.^{30–32} To identify candidate therapeutic targets in the chromatin-regulatory network in acute myeloid leukemia (AML), we have recently performed a multiplexed short hairpin RNA (shRNA) screen in a genetically engineered mouse model of AML.³² In this screen, we identified the 'epigenetic reader' bromodomain-containing protein-4 (BRD4) as a promising new target in AML.³² Thus, inhibition of BRD4 by exposure to RNA interference or treatment with JQ1, a drug blocking the specific interactions between BRD4 and acetylated histones, resulted in major antileukemic effects in murine and human AML cells.^{32,33}

The aims of the present study were to explore whether neoplastic MCs in MCL and other types of SM express BRD4 and

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whether BRD4 serves as a druggable target in these incurable malignancies.

PATIENTS AND METHODS

Reagents

A description of reagents is provided in the Supplementary Materials. A specification of monoclonal antibodies used is shown in Supplementary Table S1.

Patients and isolation of primary neoplastic MCs

Diagnostic bone marrow (BM) samples were obtained from the iliac crest of 62 patients with SM, including 46 with ISM (including one with smoldering subtype of SM), 8 with ASM, 5 with SM-AHNMD and 3 with MCL. Diagnoses were established according to WHO criteria.^{6–8} Patient characteristics are shown in Supplementary Table S2. BM aspirates were collected in syringes containing preservative-free heparin. All patients gave written informed consent before BM examination. The study was approved by the ethics committee of the Medical University of Vienna. Aspirated BM cells were layered over Ficoll to isolate mononuclear cells. Mononuclear cells were examined for drug responses, expression of BRD4 mRNA and presence of *KIT* mutations at codon 816 as reported.^{21,34} In one patient with MCL, a *KIT* D816H mutation was found. In most other patients, neoplastic cells were found to carry *KIT* D816V (Supplementary Table S2). Control samples (normal/reactive BM) were from patients with idiopathic cytopenia ($n=2$), lymphomas without BM infiltration ($n=6$), autoimmune thyroiditis ($n=1$) and chronic myeloid leukemia in major molecular response ($n=1$).

Cell lines

The MCL cell line HMC-1³⁵ was kindly provided by Dr JH Butterfield (Mayo Clinic, Rochester, MN, USA). Two subclones were used, HMC-1.1 expressing *KIT* V560G and HMC-1.2 harboring *KIT* V560G and *KIT* D816V.^{24,36} HMC-1 cells were maintained in Iscove's modified Dulbecco's medium containing 10% fetal calf serum, L-glutamine and antibiotics (at 37 °C with 5% CO₂). The recently established human MC lines ROSA^{KIT} WT and ROSA^{KIT} D816V were cultured in Iscove's modified Dulbecco's medium with 10% fetal calf serum.³⁷ ROSA^{KIT} WT cells were maintained in stem cell factor (SCF), and ROSA^{KIT} D816V cells were maintained without SCF. Chinese hamster ovary cells transfected with the murine *SCF* gene served as a source of SCF.³⁷ In select experiments, recombinant human SCF (rhSCF) was used. The identity of the HMC-1 and ROSA cell lines was confirmed by mutation analysis and phenotyping. For knockdown experiments, pRRL-SFFV-GFP-mir-E was constructed based on pRRL-SGEP³⁸ by removing the PGK-Puro cassette. Knockdown-validated shRNAs targeting *BRD4* or *Renilla* luciferase (Supplementary Table S3) were cloned using *XhoI/EcoRI* from existing miR-E constructs. Lentivirus was produced by transfection of HEK-293FT cells with shRNA constructs and third-generation lentiviral packaging vectors (pRSV-Rev; Addgene no. 12253; pMD2.G, Addgene no. 12259; Addgene, Cambridge, MA, USA; and pcDNA3.GP4xCTE, kindly provided by Dr A Schambach, Hannover Medical School, Hannover, Germany) as described previously.³⁸ HMC-1 and ROSA cells were transduced using spin infection (800 g for 90 min at 32 °C) in the presence of polybrene (7 µg/ml). GFP+ cells were sorted on a FACSAria (Becton Dickinson Biosciences, San Jose, CA, USA).

Immunocytochemistry and immunohistochemistry

To study the expression of BRD4 in neoplastic MCs, immunocytochemistry and immunohistochemistry were performed according to published protocols.^{39,40} A description of staining techniques used in this study is provided in the Supplementary Material.

Evaluation of MC proliferation

Primary cells (mononuclear cells, 10×10^4 cells per well) and cell lines ($1-2 \times 10^4$ cells per well) were cultured in 96-well microtiter plates in RPMI-1640 medium plus 10% fetal calf serum in the absence or presence of JQ1 (5–5000 nM) at 37 °C. After 48 h, ³H-labeled thymidine (0.5 µCi per well) was applied for 16 h. Cells were then harvested in filter membranes and bound radioactivity was measured in a β-counter. In a separate set of experiments, drugs were applied in the absence or presence of either Chinese hamster ovary supernatant containing SCF or rhSCF (200 ng/ml)

for 24 h. In another set of experiments, drug combinations were tested, using JQ1 and other drugs (PKC412, cladribine, 5-azacytidine, decitabine, ATRA (all-trans retinoic acid)). After an initial screen, PKC412 and ATRA were identified as potent drug partners. HMC-1 cells were incubated with suboptimal concentrations of JQ1 and ATRA or JQ1 and PKC412 at a fixed ratio of drug concentrations. Synergism was defined as supra-additive effect that was confirmed by calculating combination index (CI) values using Calcsyn software (Calcsyn Biosoft, Ferguson, MO, USA) as reported.^{24,26}

Assessment of apoptosis and cell cycle progression in neoplastic MCs

A detailed description of apoptosis evaluation techniques and cell cycle progression analysis is provided in the Supplementary Materials.

Evaluation of the expression of activation antigens in drug-exposed cells

Recent data suggest that JQ1 induces maturation in AML cells.^{32,33} To study the effects of JQ1 on MC differentiation, flow cytometry experiments were performed. HMC-1.1, HMC-1.2, ROSA^{KIT} WT and ROSA^{KIT} D816V cells were incubated in JQ1 (100–5000 nM) at 37 °C for 48 h. Then, viable cells were examined for the expression of CD2 (LFA-2), CD9, CD11b (C3biR), CD54 (ICAM-1), CD63 (LAMP-3), CD71 (transferrin receptor), CD95 (FAS), CD117 (KIT), CD203c (E-NPP3) and FcεR1α by flow cytometry on a FACSCalibur. In addition, cells were examined for morphologic signs of maturation by Wright–Giemsa staining.

Histamine release experiments

Histamine release experiments were performed on ROSA^{KIT} WT cells following a standard protocol.^{41,42} A description of the histamine release assay is provided in the the Supplementary Materials.

Quantitative PCR

A detailed description of quantitative PCR experiments is provided in the Supplementary Materials. Primers used in PCR assays are shown in Supplementary Table S4.

Statistical analysis

Differences in growth and apoptosis in drug-exposed cells were determined by appropriate statistical analysis, including analysis of variance and the Student's *t*-test. Results were considered to be significantly different when the *P*-value was < 0.05.

RESULTS

Cytoplasmic BRD4 is expressed abundantly in neoplastic MCs in ASM and MCL

In all 26 SM patients tested, neoplastic MCs were found to exhibit nuclear BRD4 (Figure 1a and Supplementary Table S2). In ASM and MCL, neoplastic MCs also expressed substantial amounts of cytoplasmic BRD4 (Figure 1a). By contrast, in most patients with ISM, MCs expressed only little if any cytoplasmic BRD4 (Figure 1a and Supplementary Table S2). We were also able to show that MCs obtained from patients with advanced SM express BRD4 mRNA (Figure 1b). The MC lines HMC-1.1, HMC-1.2, ROSA^{KIT} WT and ROSA^{KIT} D816V also expressed BRD4 at the mRNA and protein levels (Figure 1b and Supplementary Figure S1). Finally, we confirmed the expression of cytoplasmic BRD4 in primary neoplastic MCs in advanced SM (Supplementary Figure S1). Preincubation of anti-BRD4 antibody with a BRD4-specific blocking peptide or antibody omission resulted in a negative stain (Supplementary Figure S1). Neither normal CD34+ progenitor cells nor SCF-dependent cultured (non-neoplastic) MCs expressed substantial amounts of BRD4 (Supplementary Figure S1).

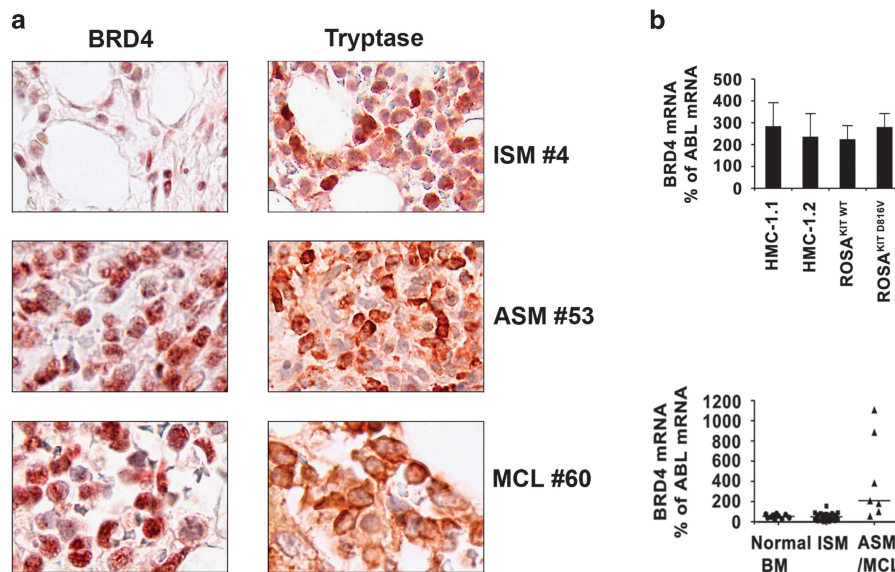


Figure 1. Expression of BRD4 in neoplastic MCs. **(a)** BM sections obtained from patients with ISM (upper panels), ASM (middle panels) or MCL (lower panels) were stained with antibodies against BRD4 (left panels) or tryptase (right panels) as described in the text. Tryptase was used to detect MCs. Original magnification: $\times 100$. #: Patient details are listed in Supplementary Table S2. **(b)** BRD4 mRNA expression in HMC-1 and ROSA cells (upper panel), in normal BM cells ($n=10$ donors) and in mononuclear BM cells obtained from patients with ISM ($n=42$) or advanced SM (ASM/MCL) ($n=7$) (lower panel). Quantitative PCR (qPCR) was performed as described in the text. Results are expressed as the percent of ABL mRNA and represent the mean \pm s.d. of five independent experiments in cell lines (upper panel) and median values (indicated by horizontal bars) for each group of donors. As assessed by analysis of variance (ANOVA) and unpaired t-test, BRD4 mRNA expression was significantly higher in the ASM/MCL group compared with normal donors or ISM ($P < 0.05$).

Regulation of expression of BRD4 in neoplastic MCs

SCF was found to upregulate BRD4 mRNA expression in ROSA^{KIT D816V} but not in HMC-1 cells (Supplementary Figure S2). To explore the underlying signaling pathways, we applied KIT-targeting drugs and a panel of signal-transduction inhibitors. As shown in Supplementary Figures S2 and S3, the KIT-targeting drugs PKC412 and dasatinib dose-dependently suppressed the expression of BRD4 mRNA in all cell lines tested (Supplementary Figure S3). Imatinib was found to suppress BRD4 mRNA expression in HMC-1.1 and ROSA^{KIT WT} cells but not in HMC-1.2 or ROSA^{KIT D816V} cells (Supplementary Figure S2). These data are in line with the notion that KIT D816V confers resistance against imatinib.^{17,18} The mitogen-activated protein kinase kinase (MEK) inhibitors RDEA119 and PD0325901, the PI3K/mTOR (phosphoinositide 3-kinase/mammalian target of rapamycin) blocker BEZ235 and ATRA were also found to downregulate BRD4 expression in HMC-1 and ROSA cells. Rapamycin induced a slight decrease in BRD4 mRNA expression in HMC-1.2 and ROSA^{KIT D816V} cells but not in ROSA^{KIT WT} or HMC-1.1 cells. BRD4 mRNA expression remained unchanged after exposure to the other drugs examined, including the signal transducer and activator of transcription 5 blockers piceatannol and pimozide, cladribine (2CdA), and the demethylating agents, 5-azacytidine and decitabine. INCB018424 (ruxolitinib) slightly reduced the expression of BRD4 mRNA in ROSA^{KIT D816V} and HMC-1.2 cells (Supplementary Figure S2).

The BRD4-targeting drug JQ1 inhibits the proliferation of neoplastic MCs

We have recently shown that shRNAs against BRD4 and the BRD4-targeting drug JQ1 inhibit the growth of AML cells.^{32,33} To demonstrate a role for BRD4 as potential target in neoplastic MCs, we applied BRD4-specific shRNAs. BRD4 knockdown was confirmed by quantitative PCR. As visible in Figure 2a and Supplementary Figures S4A and S4B, the shRNA-induced knockdown of BRD4 resulted in a significant growth inhibition of HMC-1 and ROSA cells. The knockdown of BRD4 was also found to

decrease MYC mRNA levels in all cell lines (not shown). JQ1 was found to inhibit ³H-thymidine uptake in HMC-1.1 and HMC-1.2 cells as well as in ROSA^{KIT WT} and ROSA^{KIT D816V} cells, with IC₅₀ values of 500–1000 nM (Figures 2b and c). Similar effects were seen with volasertib and BI2536, two other drugs targeting BRD4 (Supplementary Figure S4C). JQ1 also suppressed growth in primary neoplastic MCs obtained from patients with ASM or MCL (Figure 2d). Interestingly, IC₅₀ values obtained with JQ1 in primary cells were lower compared with IC₅₀ values produced with the MC lines tested. Finally, JQ1 was found to induce a G1 cell cycle arrest in all MC lines examined (Supplementary Figure S5).

JQ1 induces apoptosis in neoplastic MCs

JQ1 was found to induce dose-dependent apoptosis in HMC-1 and ROSA cells as evidenced by light microscopy, AnnexinV staining and active caspase-3 staining (Figures 3a and b and Supplementary Figures S6A). The apoptosis-inducing effect of JQ1 was similar in HMC-1.1 cells compared with HMC-1.2 cells (Figures 3a and b and Supplementary Figure S6A) and was also comparable in ROSA^{KIT WT} and ROSA^{KIT D816V} cells (Figures 3a and b and Supplementary Figure S6A). We were also able to confirm the apoptosis-inducing effects of JQ1 on HMC-1 and ROSA cells in a terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (Supplementary Figure S6B). Finally, we were able to show that shRNAs against BRD4 induce apoptosis in HMC-1 and ROSA cells (not shown).

JQ1 downregulates the expression of CD63, CD71 and FcεRI in neoplastic MCs

Recent data suggest that JQ1 can induce maturation in AML cells.^{32,33} In the present study, we found that JQ1 dose-dependently downregulates the expression of CD63 and CD71 in HMC-1 and ROSA cells (Supplementary Figure S7A) and HMC-1.2 cells. In ROSA^{KIT WT} and ROSA^{KIT D816V} cells, JQ1 was also found to suppress the expression of the FcεRI (Supplementary Figure S7B). Moreover, we found that preincubation of ROSA^{KIT WT}

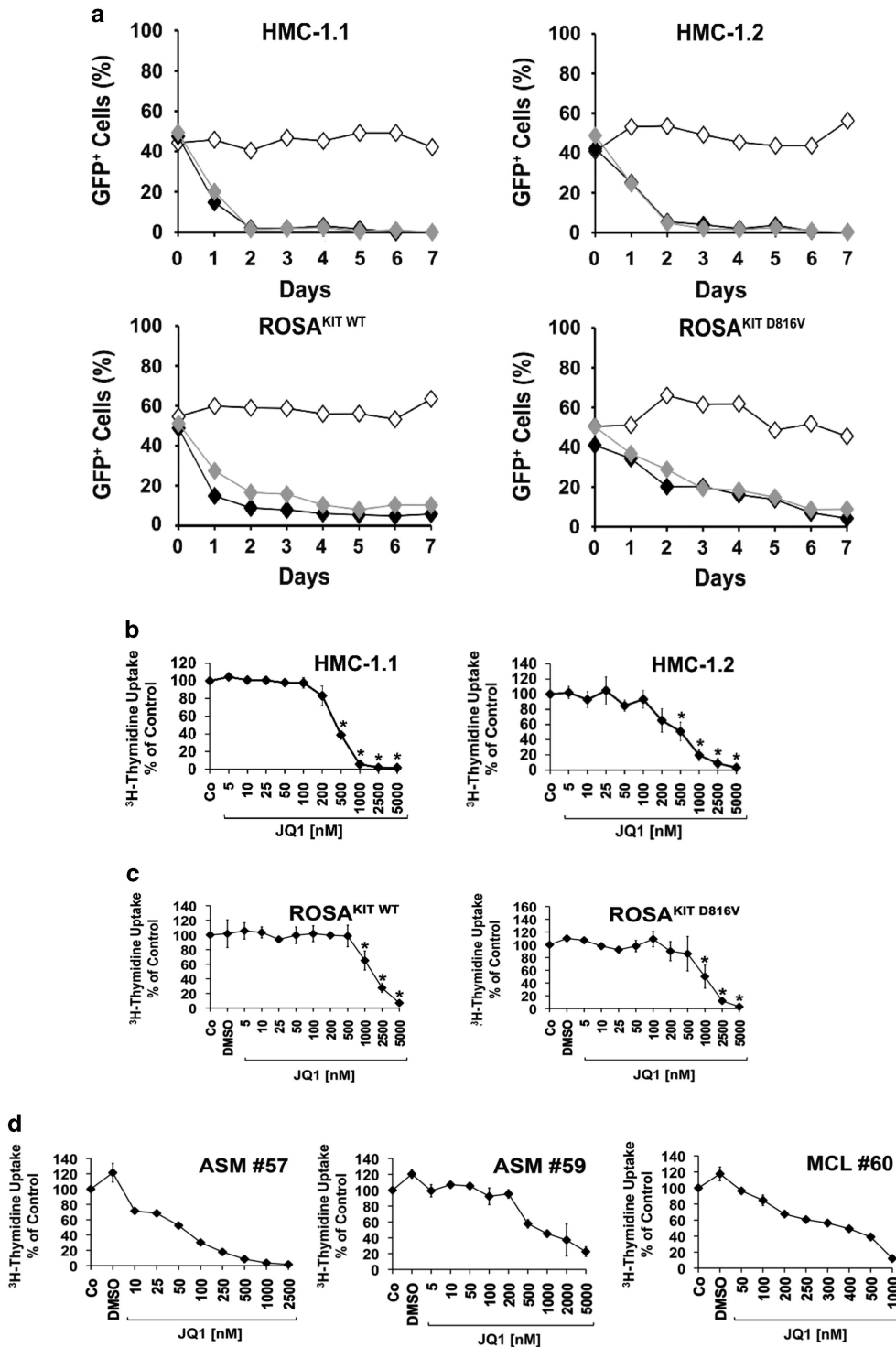


Figure 2. BRD4 shRNA and JQ1 inhibit the proliferation of neoplastic MCs. **(a)** Effect of shRNA-mediated knockdown of BRD4 on proliferation of HMC-1.1, HMC-1.2, ROSA^{KIT} WT and ROSA^{KIT} D816V cells. Cells were transfected with a control shRNA targeting *Renilla* luciferase (◇-◇) or with two different shRNA constructs targeting BRD4, #602 (◆◆) and #1817 (◆◆). GFP+ cells were sorted, mixed with their non-transduced control cells (ratio 1:1) and maintained in culture for 7 days. The percentage of GFP+ cells was measured daily by flow cytometry. Results show the percentage of GFP+ cells in one typical experiment. **(b-d)** Effects of JQ1 on proliferation of HMC-1 cells **(b)**, ROSA cells **(c)** and primary neoplastic MCs obtained from patients with ASM or MCL **(d)**. Cells were incubated in the absence (Co) or presence of various concentrations of JQ1 at 37 °C for 48 h. Then, ³H-thymidine uptake was measured. Results are expressed as the percent of control and represent the mean ± s.d. of three independent experiments **(b and c)** or the mean ± s.d. of triplicates **(d)**. **P* < 0.05 compared with control (Co).

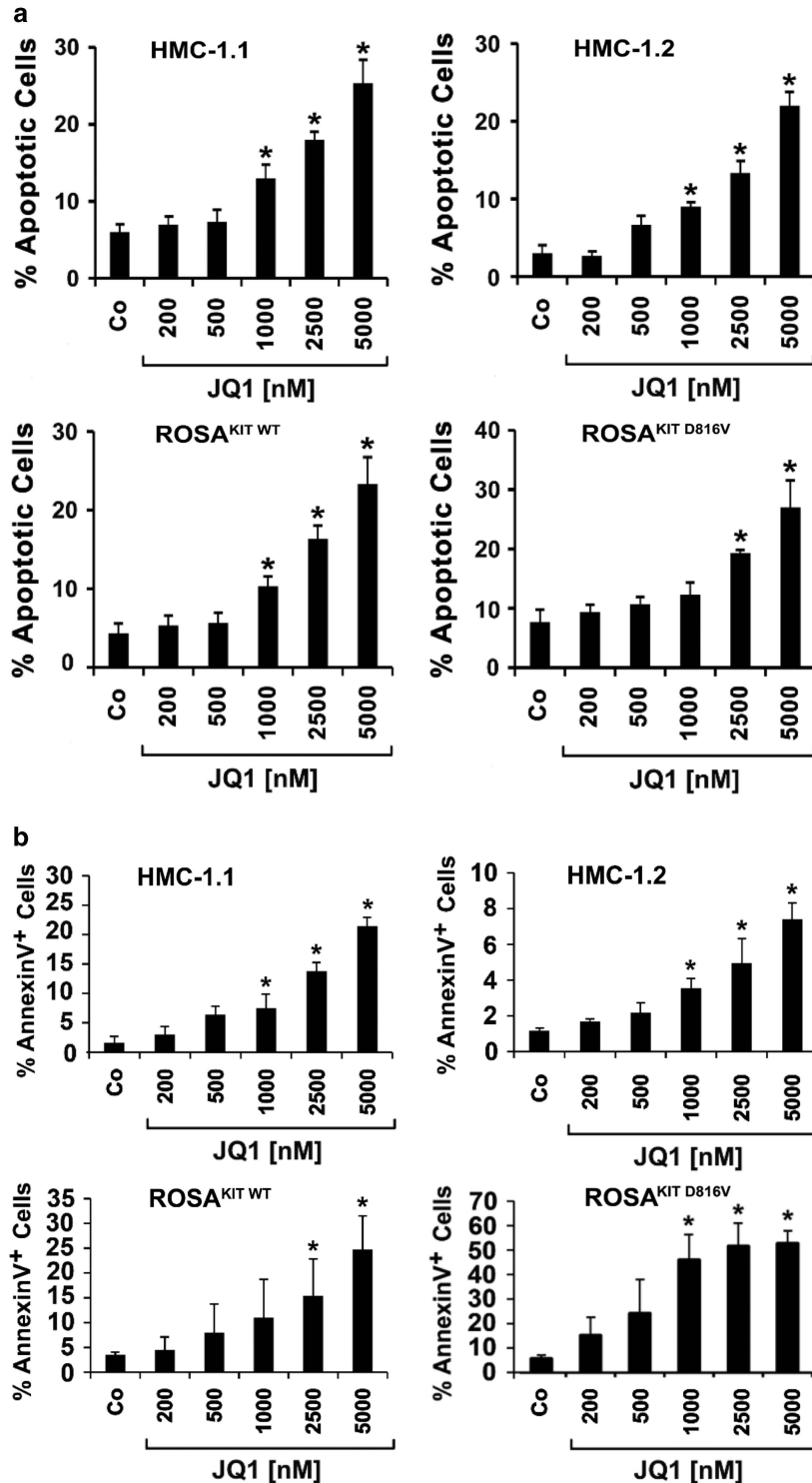


Figure 3. JQ1 induces apoptosis in neoplastic MCs. (a) HMC-1 and ROSA cells were incubated in the absence (Co) or presence of various concentrations of JQ1 at 37 °C for 48 h. Thereafter, the percentage of apoptotic cells was determined by light microscopy. Results show the percent of apoptotic cells and represent the mean \pm s.d. of three independent experiments. (b) After incubation in control medium (Co) or JQ1, HMC-1 and ROSA cells were stained with AnnexinV/propidium iodide (PI), and the percentage of apoptotic cells was determined by flow cytometry. Results show the percent of AnnexinV/PI⁺ cells and represent the mean \pm s.d. of three independent experiments. * $P < 0.05$ compared with control.

cells with JQ1 blocks IgE-mediated histamine release (Supplementary Figure S8) and counteracts IgE-mediated upregulation of CD63 and CD203c (Supplementary Figure S9). Although JQ1 alone failed to upregulate the expression of differentiation antigens on neoplastic MCs, the drug combination 'JQ1+ATRA' was found to upregulate the

expression of CD11b (C3b1R) and CD54 (ICAM-1) in HMC-1 and ROSA cells (Supplementary Figure S10A). By contrast, when used as a single agent, ATRA did not upregulate CD54 or CD11b in HMC-1 or ROSA cells (not shown). ATRA also failed to modulate the expression of CD63 and CD71, and neither JQ1 nor ATRA (as single agents or in

combination) produced visible signs of maturation in HMC-1 or ROSA cells (Supplementary Figure S10B).

Effects of JQ1 on the expression of MYC and BCL-2 family members in MCs

Recent data suggest that JQ1 modulates the expression of MYC and various BCL-2 family members in leukemic cells.^{43–45} We found that JQ1 downregulates the expression of BCL-2, MCL-1 and BCL-xL mRNA levels, as well as MYC mRNA expression levels, in HMC-1 and ROSA cells (Supplementary Figure S11). In addition, we found that JQ1 upregulates BIM mRNA levels in all four cell lines tested (Supplementary Figure S11).

JQ1 synergizes with PKC412 and with ATRA in producing growth inhibition in HMC-1 and ROSA cells

As BRD4-targeting drugs may best be applied in combination with other antineoplastic drugs in patients with advanced SM, we screened for suitable drug partners of JQ1. A number of candidates, including cladribine (2CdA), 5-azacytidine and decitabine, did not show synergistic effects with JQ1 (not shown). However, we found that JQ1 strongly synergizes with PKC412 and with ATRA in suppressing the growth of HMC-1 and ROSA cells (Supplementary Figure S12A). In addition, JQ1 was found to synergize with PKC412 and ATRA in inducing apoptosis in HMC-1 and ROSA cells (Supplementary Figures S12B and S12C). Synergism was confirmed by calculating CI values (< 1) (not shown).

KIT activation confers resistance against JQ1 in ROSA^{KIT D816V} cells
In a final step, we screened for potential resistance mechanisms that could interfere with JQ1 effects. As KIT has been implicated in MYC activation and upregulates BRD4 expression, we asked whether KIT activation could interfere with JQ1 effects. Indeed, as visible in Figures 4a and b, incubation of ROSA^{KIT D816V} cells with SCF blocked their responsiveness against JQ1. Interestingly, however, SCF did not alter responsiveness of HMC-1.1 and HMC-1.2 cells to JQ1 (Supplementary Figure S13A). We also asked whether the combination 'JQ1+PKC412' would overcome KIT-mediated resistance. Indeed, this drug combination was found to produce strong synergistic apoptosis-inducing effects in the presence of SCF in ROSA^{KIT D816V} cells (Supplementary Figure S13B).

DISCUSSION

Bromodomain-containing proteins have recently been identified as key regulators of cell cycle progression and growth in various neoplastic cells.^{29–33} One of these regulators appears to be BRD4, an epigenetic reader involved in the regulation of MYC. We have

recently shown that BRD4 is a druggable target in AML cells,^{32,33} and similar observations have been made in other tumor models.^{43–45} We here show that BRD4 is abundantly expressed in neoplastic MCs in advanced SM, and that the BRD4-targeting drug JQ1 induces growth inhibition and apoptosis in these cells.

ASMs and MCLs are incurable malignancies characterized by drug resistance and a poor survival.^{1–6,10–15} In these patients, neoplastic cells are often resistant against conventional drugs and KIT-targeting tyrosine kinase inhibitors. Therefore, it is important to define new robust targets in these malignancies. We have identified BRD4 as a novel drug target in neoplastic MCs in advanced SM. To the best of our knowledge, this is the first report describing a BET-bromodomain-containing molecule as a target in neoplastic MCs.

We have recently shown that BRD4 is expressed abundantly in the cytoplasm of leukemic blasts in AML.³³ In the present study, we show that BRD4 is expressed abundantly in the cytoplasm and the nuclei of neoplastic MCs in almost all patients with advanced SM, whereas in most patients with ISM, MCs expressed only low levels or no detectable BRD4 in their cytoplasm but still expressed BRD4 in their nuclei. HMC-1 and ROSA cells also expressed cytoplasmic and nuclear BRD4. The abundant expression of cytoplasmic BRD4 in MCs in advanced SM may have several explanations. One hypothesis is that cytoplasmic expression of BRD4 is indicative of a higher proliferative potential of neoplastic cells. Alternatively, KIT-independent lesions acquired during disease progression promote cytoplasmic BRD4 expression. Indeed, apart from KIT D816V, several additional molecular lesions and pro-oncogenic signaling pathways are expressed in neoplastic MCs in advanced SM.^{46,47} The exact role and biochemical function of cytoplasmic BRD4 in neoplastic MCs remain unknown. However, there is evidence that BRD4 exerts kinase activity and interacts with cytoplasmic antigens, such as SPA-1, in malignant cells.^{48–50} Whether BRD4 also fulfills these functions in neoplastic MCs remains unknown.

So far, little is known about the mechanisms contributing to the expression of BRD4 in neoplastic MCs. In an attempt to define signaling pathways potentially involved in the expression of BRD4, we applied various signal-transduction inhibitors. The multi-targeted tyrosine kinase inhibitors PKC412 and dasatinib, which are known to block the kinase activity of KIT D816V, were found to downregulate the expression of BRD4 in HMC-1 and ROSA cells. In addition, we found that various MEK inhibitors and the PI3K/mTOR blocker BEZ235 can downregulate the expression of BRD4 in neoplastic MCs. As MEK as well as PI3K are downstream of KIT, these data suggest that KIT D816V contributes to the expression of BRD4 in neoplastic MCs. This conclusion was supported by the observation that SCF promotes the expression of BRD4 in ROSA^{KIT D816V} cells. However, SCF failed to upregulate the

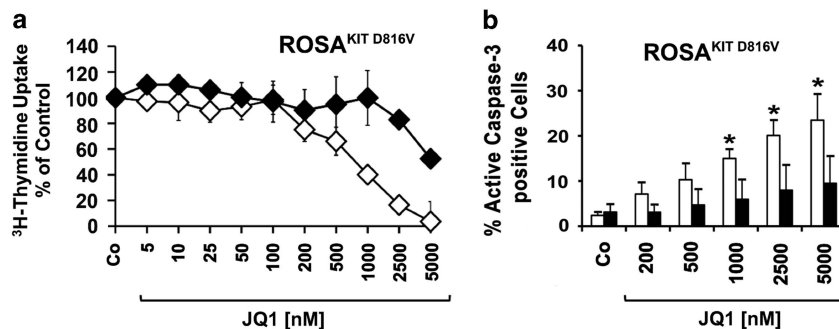


Figure 4. Effects of SCF on responsiveness of neoplastic MCs to JQ1. (a and b) ROSA^{KIT D816V} cells were incubated in the absence (◇-◇ in the left image; open bars in the right image) or presence of rhSCF (200 ng/ml) (◆-◆ in the left image; black bars in the right image) at 37 °C for 24 h. Thereafter, cells were washed and incubated in control medium (Co) or in various concentrations of JQ1 (5–5000 nM) for 48 h. Then, ³H-labeled thymidine uptake (left panel) and the percentage of active caspase-3-positive cells (right panel) were measured. Results represent the mean ± s.d. of three independent experiments. *P < 0.05 compared with Co.

expression of BRD4 in HMC-1 cells. These observations suggest that the expression of BRD4 in MCs depends not only on KIT activation but also on other factors. An interesting observation was that BRD4 mRNA expression levels are slightly higher in HMC-1.1 compared with that in HMC-1.2 cells. The reason for this phenomenon remains unknown.

We have recently shown that the BRD4-targeting drug JQ1 exerts strong antineoplastic effects in drug-resistant AML cells.^{33,34} In the present study, we were able to show that JQ1 induces growth inhibition and apoptosis in primary neoplastic cells obtained from patients with advanced SM as well as in HMC-1 and ROSA cells. In each cell line tested, the effects of JQ1 were dose-dependent, with reasonable IC₅₀ values, and in each case, growth inhibition was associated with induction of apoptosis.

In AML, JQ1 effects were found to be associated with signs of maturation.^{32,33} We were therefore interested to learn whether exposure to JQ1 would lead to maturation in neoplastic MCs. However, we were not able to detect a morphologically visible maturation-inducing effect of JQ1 in HMC-1 or ROSA cells. Moreover, we were unable to demonstrate that JQ1 induces the upregulation of differentiation-associated antigens in MCs. Rather, JQ1 was found to even downregulate the expression of several key surface antigens in HMC-1 and/or ROSA cells, including CD63 and CD71. Furthermore, JQ1 was found to downregulate the expression of FcεRI and to suppress IgE-dependent histamine release in ROSA cells. Collectively, these data suggest that JQ1 blocks proliferation as well as activation of neoplastic MCs, which may be relevant clinically, as patients with advanced SM not only suffer from organ infiltration but also from the consequences of MC activation and mediator release.^{2–6}

The intriguing effects of JQ1 on growth and survival of neoplastic MCs prompted us to ask for optimal drug partners and synergistic antineoplastic effects. Of all drug partners tested, PKC412 and ATRA were found to synergize with JQ1 in inhibiting growth and survival in HMC-1 and ROSA cells.

In a final step, we asked whether resistance against JQ1 may occur in neoplastic MCs. In these experiments, we found that SCF introduces resistance against JQ1 in ROSA^{KIT D816V} cells but not in HMC-1 cells. We also asked whether the synergistic activity of the drug combinations JQ1+ATRA and JQ1+PKC412 would overcome SCF-mediated resistance. Indeed, the combination JQ1+PKC412 was found to overcome SCF-mediated resistance in ROSA, whereas the combination JQ1+ATRA was unable to overcome resistance. This is best explained by the fact that SCF-induced resistance is mediated by the activation of KIT, the principle target of PKC412.

In summary, we have shown that BRD4 is a druggable target in neoplastic MCs. Inhibition of BRD4 by JQ1 is a potent approach to eradicate neoplastic MCs, and drug effects can be potentiated by the addition of PKC412 or ATRA. Whether BET-targeting drugs exert beneficial effects in patients with advanced SM remains to be determined.

CONFLICT OF INTEREST

JEB is a scientific founder of Tensha Therapeutics, which is developing drug-like derivatives of the JQ1 bromodomain inhibitor as cancer therapeutics, through a license from the Dana-Farber Cancer Institute. H-PH has a consultancy with and received honoraria from Novartis. PV has a consultancy with and received honoraria and research funding from Novartis. The authors declare no other conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

Identification of bromodomain-containing protein 4 (BRD4) as a novel marker and epigenetic target in mast cell leukemia

Materials and Methods

Reagents

RPMI 1640 medium was purchased from Lonza (Basel, Switzerland), Iscove's Modified Dulbecco's Medium (IMDM) and fetal calf serum (FCS) from Gibco Life Technologies (Carlsbad, CA, USA), recombinant human (rh) stem cell factor (SCF) from Peprotech (Rocky Hill, NJ, USA), ³H-thymidine from PerkinElmer (Waltham, MA, USA), and 5-azacytidine, decitabine, all-trans-retinoic acid (ATRA), rapamycin, pimozide, piceatannol and 2CdA (cladribine) from Sigma Aldrich (St. Louis, MO, USA). PKC412 (midostaurin) was kindly provided by Dr.P.W.Manley, Novartis Basel (Switzerland) or purchased from LC Laboratories (Woburn, MA, USA). Ponatinib was purchased from Selleck (Houston, TX, USA). Dasatinib, imatinib, the MEK inhibitors RDEA119 and PD0325901, the JAK2 blockers TG101348 and INCB018424 (ruxolitinib), the PI3K/mTOR inhibitor BEZ235, and volasertib and BI2536 were purchased from Chemietek (Indianapolis, IN, USA). JQ1 was synthesized as described previously.¹ All-trans retinoic acid (ATRA) was dissolved in ethanol, imatinib in water, and all other drugs in dimethylsulfoxide (DMSO) (Sigma Aldrich). A specification of monoclonal antibodies (mAb) used in this study is shown in Supplementary Table S1.

Immunocytochemistry (ICC) and immunohistochemistry (IHC)

To study the expression of BRD4 in neoplastic mast cells (MCs), ICC and IHC were performed according to published protocols.^{2,3} ICC was performed on HMC-1 cells, ROSA cells, normal human CD34+ progenitor cells, normal stem cell factor (SCF)-cultured peripheral blood (PB)-derived MCs and primary neoplastic MCs obtained from patients with indolent systemic mastocytosis (ISM) (n=5), aggressive systemic mastocytosis (ASM) (n=1) or mast cell leukemia (MCL) (n=2). Cells were spun on cytospin-slides and incubated with a polyclonal rabbit anti-BRD4 antibody (Sigma-Aldrich; 1:20) at 4°C for 20 hours. Cells were then washed and incubated with biotinylated goat-anti-rabbit IgG for 30 minutes. Alkaline phosphatase complex (Biocare, Walnut Creek, CA, USA) was used as chromogen. In control experiments, the anti-BRD4 antibody was preincubated with a BRD4-specific blocking peptide (Bethyl Lab, Montgomery, TX, USA). IHC was performed on sections prepared from paraffin-embedded BM biopsy specimens (ISM, n=15; SM-AHNMD, n=4; ASM, n=5; MCL, n=2; Table 1) using the indirect immunoperoxidase staining technique as reported.^{2,3} Prior to staining, sections were pretreated by microwave oven. Slides were incubated with anti-BRD4 antibody (1:20; pH 7.5) at 4°C for 20 hours. Then, slides were washed and incubated with biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) for 30 minutes, washed, and then exposed to Vectastain ABC KIT (30 minutes). AEC (Sigma Aldrich) was used as chromogen. Stained sections were analyzed by Olympus AX-1 microscope equipped with a 60x/0.9 UPlan-Apo objective lens. Images were taken on an Olympus DP21 camera (Olympus, Hamburg, Germany) and adjusted by Adobe Photoshop CS2 software Version 9.0 (Adobe Systems, San Jose, CA, USA).

Assessment of apoptosis and cell cycle progression in neoplastic MCs

HMC-1 and ROSA cells were incubated in control medium or in various concentrations of JQ1 at 37°C for 48 hours. Then, cells were subjected to flow cytometry following published protocols.³⁻⁵ Apoptosis was measured by combined AnnexinV/propidium-iodide staining or staining for active caspase-3.^{3,5} For staining of caspase-3, cells were fixed in 2% paraformaldehyde and permeabilized in methanol at -20°C (15 minutes). Rabbit anti-human mAb C92-605 against active caspase-3 was applied for 30 minutes. Then, cells were washed and analyzed on a FACSCalibur (Becton Dickinson Biosciences). Drug-induced apoptosis was also measured on Wright-Giemsa-stained cytopsin slides prepared from HMC-1 and ROSA cells. In select experiments, drug-induced apoptosis (5,000 nM JQ1 for 48 hours) was confirmed by TUNEL Assay as described previously.⁴ Cell cycle analysis was performed with HMC-1 and ROSA cells after exposure to control medium or JQ1 (250-2,500 nM) at 37°C for 24 hours. Cell cycle progression was analyzed by flow cytometry as reported.³

Quantitative PCR (qPCR)

qPCR was performed with RNA isolated from HMC-1 cells, ROSA cells, normal donors or primary neoplastic MCs. Cell lines were incubated in control medium or JQ1 (2,500 nM) for 24 hours. In select experiments cells were treated with different targeted drugs for 12 hours. Then, RNAs were isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). BRD4, BCL-2, MCL-1, BCL-xL, BIM, and MYC mRNA levels were quantified on a 7900HT Fast Real-Time PCR System (Applied

Biosystems, Foster City, CA), using iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA) following published protocols.^{3,6} Primers used in PCR assays are shown in Supplementary Table S3. Results were expressed as percent of ABL mRNA levels.

Histamine release experiments

Histamine release experiments were performed on ROSA^{KIT WT} cells following a standard protocol.^{7,8} Cells were incubated with IgE (10 µg/ml) (Calbiochem, Darmstadt, Germany) and IL-4 (10 ng/ml, Peprotech) in IMDM at 37°C for 80 hours. Then, cells were split and incubated in control medium or in medium containing JQ1 (200 nM) at 37°C overnight. Cells were then washed and exposed to 3 different concentrations of anti-IgE antibody E-124.2.8 (0.1, 1, and 10 µg/ml) in histamine release buffer (HRB, Immunotech, Marseilles, France) at 37°C for 30 minutes. Thereafter, cells were centrifuged at 4°C and the cell-free supernatants and cell suspensions were recovered and examined for histamine content by radioimmunoassay (RIA) (Immunotech). Histamine release was calculated as percent of released (extracellular) histamine relative to total (cellular+extracellular) histamine.^{7,8}

Supplementary Tables

Supplementary Table S1

Specification of antibodies used in immunostaining experiments

CD	Antigen	Clone	Fluorochrome		Manufacturer*
			Conjugate	Species, Isotype	
CD2	LFA-2	RPA-2.10	PE	Mouse, IgG1	BD Biosciences
CD9	TSPAN-29/p24	M-L13	PE	Mouse, IgG1	BD Biosciences
CD11b	C3biR	ICRF44	PE	Mouse, IgG1	Biologend
CD44	Pgp1	515	PE	Mouse, IgG1	BD Biosciences
CD54	ICAM-1	HA58	PE	Mouse, IgG1	BD Biosciences
CD63	LAMP-3	CLB-gran12	PE	Mouse, IgG1	BD Biosciences
CD71	Tf-R	CY1G4	PE	Mouse, IgG1	Biologend
CD95	FAS	DX2	PE	Mouse, IgG1	BD Biosciences
CD117	KIT	104D2	PE	Mouse, IgG1	BD Biosciences
CD164	Sialomucin	67D2	PE	Mouse, IgG1	Biologend
CD203c	E-NPP3	97A6	PE	Mouse, IgG1	BeckmanCoulter
n.c.	FcεR1α	AER-37	APC	Mouse, IgG1	Biologend
n.c.	Isotype control	MOPC-21	PE	Mouse, IgG1	BD Biosciences
n.c.	Isotype control	G155-178	FITC	Mouse, IgG2a	BD Biosciences
n.c.	Isotype control	MOPC-21	APC	Mouse, IgG2b	BD Biosciences
n.c.	Tryptase	G3	-	Mouse, IgG1	Chemicon
n.c.	BRD4	Polyclonal	-	Rabbit, IgG	Sigma-Aldrich
n.c.	BRD4	Polyclonal	-	Rabbit, IgG	Bethyl Labs

CD, cluster of differentiation; n.c, not clustered; LFA-2, leukocyte function antigen-2; TSPAN-29/p24, tetraspanin-29/platelet antigen p24; C3biR, complement receptor 3; Pgp1, phagocyte glycoprotein 1; ICAM-1, intercellular adhesion molecule 1; LAMP-3, lysosome-associated membrane glycoprotein 3; Tf-R, transferrin receptor; FAS, Apoptosis stimulating fragment; E-NPP3, ectonucleotide pyrophosphatase/phosphodiesterase 3; FcεR1α; IgE receptor alpha chain; BRD4, bromodomain-containing protein 4; PE, phycoerythrin; APC, allophycocyanin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; BD, Becton Dickinson.

*Manufacturers' locations: Becton Dickinson: San Jose, CA, USA; Biologend: San Diego, CA, USA; Immunotech: Marseille, France; Beckman Coulter: Brea, CA, USA; Chemicon, Temecula, CA, USA; Sigma-Aldrich: St. Louis, MO, USA; Bethyl Labs: Montgomery, TX, USA.

Supplementary Table S2
Patients' characteristics and expression of BRD4 in neoplastic mast cells (MCs)

No#	Gender	Age yrs	Diagnosis	Karyotype	Tryptase (ng/ml)	<i>KIT</i> Mutation	% MCs BM section	% MCs BM smear	BRD4* nuclear	BRD4* cytoplasmic
1	m	52	ISM	n.d.	22.5	<i>KIT</i> D816V-	5%	<1%	+	+/-
2	m	43	ISM	47,XY,+Y	79.8	<i>KIT</i> D816V+	20%	<1%	+	-/+
3	f	60	ISM	46,XX	188	<i>KIT</i> D816V+	40%	1%	+	-
4	f	47	ISM	46,XX	69.9	<i>KIT</i> D816V+	15%	<1%	+	-/+
5	m	42	ISM	46,XY	630	<i>KIT</i> D816V+	30%	<1%	+	-/+
6	m	39	ISM	46,XY	26.5	<i>KIT</i> D816V-	<5%	<1%	+	-/+
7	f	45	ISM	46,XX	30.7	<i>KIT</i> D816V+	5%	1%	+	-/+
8	m	54	ISM	46,XY	61.1	<i>KIT</i> D816V+	5%	2%	+	-/+
9	f	35	ISM	46,XX	34.1	<i>KIT</i> D816V+	5%	<1%	+	-/+
10	m	47	ISM	46,XY	82.5	<i>KIT</i> D816V+	5%	0.5-1%	+	-/+
11	f	58	ISM	46,XX	49.6	<i>KIT</i> D816V-	<1%	<1%	+	-
12	f	36	ISM	46,XX	23.3	<i>KIT</i> D816V+	<5%	2%	n.d.	n.d.
13	f	67	ISM	46,XX	17	<i>KIT</i> D816V+	<2%	1%	n.d.	n.d.
14	f	59	ISM	46,XX	67.5	<i>KIT</i> D816V+	5-10%	1%	n.d.	n.d.
15	f	53	ISM	46,XX	14.1	<i>KIT</i> D816V+	5%	<1%	n.d.	n.d.
16	m	50	ISM	n.d.	19.7	<i>KIT</i> D816V+	2-3%	<1%	n.d.	n.d.
17	m	73	ISM	46,XY	17.6	<i>KIT</i> D816V-	<1%	<0.5%	n.d.	n.d.
18	f	22	ISM	46,XX	71.1	<i>KIT</i> D816V+	<5%	<1%	n.d.	n.d.
19	f	68	ISM	46,XX	16.8	<i>KIT</i> D816V+	<1%	<1%	n.d.	n.d.
20	f	22	ISM	46,XX	11	<i>KIT</i> D816V+	<5%	<0.5%	n.d.	n.d.
21	m	81	ISM	46,XY	30.7	<i>KIT</i> D816V+	5%	2.5%	n.d.	n.d.

22	f	46	ISM	46,XX	14.4	<i>KIT</i> D816V+	<1%	<1%	n.d.	n.d.
23	m	66	ISM	46,XY	65.4	<i>KIT</i> D816V+	15%	<1%	n.d.	n.d.
24	f	63	ISM	46,XX	32.7	<i>KIT</i> D816V+	1%	<1%	n.d.	n.d.
25	f	53	ISM	46,XX	553	<i>KIT</i> D816V+	40%	<1%	n.d.	n.d.
26	m	40	ISM	46,XY	70.8	<i>KIT</i> D816V+	10%	<1%	n.d.	n.d.
27	m	32	ISM	46,XY	36	<i>KIT</i> D816V+	5%	1%	+	-
28	f	72	ISM	46,XX,del(7)(p11.2)	192	<i>KIT</i> D816V+	30%	2%	n.d.	n.d.
29	m	50	ISM	46,XY	27.6	<i>KIT</i> D816V-	<1%	<0.5%	n.d.	n.d.
30	m	52	ISM	46,XY	16.5	<i>KIT</i> D816V+	<1%	<1%	n.d.	n.d.
31	m	49	ISM	46,XY	17.1	<i>KIT</i> D816V+	5%	<1%	n.d.	n.d.
32	m	35	ISM	46,XY	60.1	<i>KIT</i> D816V+	5%	2%	n.d.	n.d.
33	m	51	ISM	n.d.	60.5	<i>KIT</i> D816V+	n.d.	<1%	+	-
34	f	42	ISM	n.d.	17.2	<i>KIT</i> D816V+	<1%	<1%	n.d.	n.d.
35	f	68	ISM	46,XX	27.6	<i>KIT</i> D816V-	<1%	<1%	n.d.	n.d.
36	m	81	ISM	n.d.	122	<i>KIT</i> D816V+	5%	2%	n.d.	n.d.
37	f	52	ISM	n.d.	14	<i>KIT</i> D816V+	<1%	<1%	n.d.	n.d.
38	f	71	ISM	46,XX	23.5	<i>KIT</i> D816V-	30%	2%	n.d.	n.d.
39	m	41	ISM	46,XY	164	n.d.	15%	3%	n.d.	n.d.
40	m	57	ISM	46,XY	n.d.	n.d.	15%	<1%	n.d.	n.d.
41	m	36	ISM	n.d.	n.d.	n.d.	10%	1%	n.d.	n.d.
42	f	35	ISM	46,XX	34.1	<i>KIT</i> D816V+	5%	<1%	n.d.	n.d.
43	m	52	ISM	46,XY	40.9	<i>KIT</i> D816V+	15%	0.5-1%	+	-/+
44	m	24	ISM	46,XY	53.7	<i>KIT</i> D816V+	5%	<1%	n.d.	n.d.
45	f	68	ISM	46,XX	41	n.d.	<5%	1%	+	-
46	f	55	SSM	46,XX	133	<i>KIT</i> D816V+	20%	<1%	n.d.	n.d.
47	m	59	ISM-CMML	46,XY	143	<i>KIT</i> D816V+	10%	<1%	+	-/+
48	m	54	ISM-NHL	46,XY	290	<i>KIT</i> D816V+	25%	<1%	+	-/+
49	f	73	ISM-CMML-eo	n.d.	67.6	<i>KIT</i> D816V+	5%	n.d.	n.d.	n.d.

50	m	53	ASM-CMML	46,XY	615	<i>KIT</i> D816V+	70%	5%	+	+
51	f	54	ASM-CMML	46,XX	1510	<i>KIT</i> D816V+	20%	<1%	+	+/-
52	m	71	ASM	46,XY	97.1	<i>KIT</i> D816V+	10-15%	1%	+	+
53	m	74	ASM	46,XY	152	<i>KIT</i> D816V+	25%	<1%	+	+
54	f	59	ASM	46,XX	138	<i>KIT</i> D816V+	50%	1%	+	+/-
55	m	63	ASM	46,XY	170	<i>KIT</i> D816V+	60%	<1%	+	+
56	m	22	ASM	46,XY	52	<i>KIT</i> D816V-	3%	<1%	n.d.	n.d.
57	m	73	ASM	n.d.	789	<i>KIT</i> D816V+	95%	n.d.	n.d.	n.d.
58	m	73	ASM	46,XY	223	<i>KIT</i> D816V+	10%	<1%	n.d.	n.d.
59	m	68	ASM	46,XY	150	<i>KIT</i> D816V+	5-10%	2%	+	+/-
60	f	54	MCL	46,XX,del(7)(q22)	904	<i>KIT</i> D816H+	90%	70%	+	+
61	f	48	MCL	46,XX	533	<i>KIT</i> D816V-	70%	80%	+	+
62	f	62	MCL-AML	n.d.	547	<i>KIT</i> D816V+	70%	n.d.	n.d.	n.d.

Abbreviations: m, male; f, female; yrs, years; ISM, indolent systemic mastocytosis; CMML, chronic myelomonocytic leukemia; NHL, Non Hodgkin's Lymphoma; CMML-eo, CMML with eosinophilia; SSM, smouldering SM; ASM, aggressive SM; MCL, mast cell leukemia; AML, acute myeloid leukemia; del, deletion; n.d., not determined; BM, bone marrow. Score: +, most cells clearly positive; +/-, weakly positive in most cells; -/+, weakly positive in a smaller subset of cells; -, negative. *Nuclear and cytoplasmic expression of BRD4 in MCs was determined by immunohistochemistry using an anti-BRD4 antibody as described in the text.

Supplementary Table S3

Sequences of miR-E based BRD4- and control shRNAs used for knockdown studies in human mast cell lines*

shRNA	Sequence
Ren.713 (Renilla Luciferase) (control)	TGCTGTTGACAGTGAGCGCAGGAATTA TAATGCTTATCTATAGTGAAGCCACAG ATGTATAGATAAGCATTATAAATTCCTA TGCCTACTGCCTCGGA
BRD4.602 (human)	TGCTGTTGACAGTGAGCGACAGGACTT CAACACTATGTTTTAGTGAAGCCACAG ATGTAAAACATAGTGTGAAGTCCTGG TGCCTACTGCCTCGGA
BRD4.1817 (human)	TGCTGTTGACAGTGAGCGACAGCAGAAC AAACCAAAGAAATAGTGAAGCCACAGA TGTATTTCTTTGGTTTGTCTGCTGGTGC CTACTGCCTCGGA

Abbreviations: shRNA, short hairpin ribonucleic acid; T, thymine; G, guanine; A, adenine; C, cytosine; BRD4, bromodomain-containing protein 4.

*Shown are 97mer oligos used for standard cloning of miR-E based shRNAs. Cloning of miR-E based shRNAs was performed as described in Fellmann et al.⁹

Supplementary Table S4

Sequences of oligonucleotide primers used for qPCR

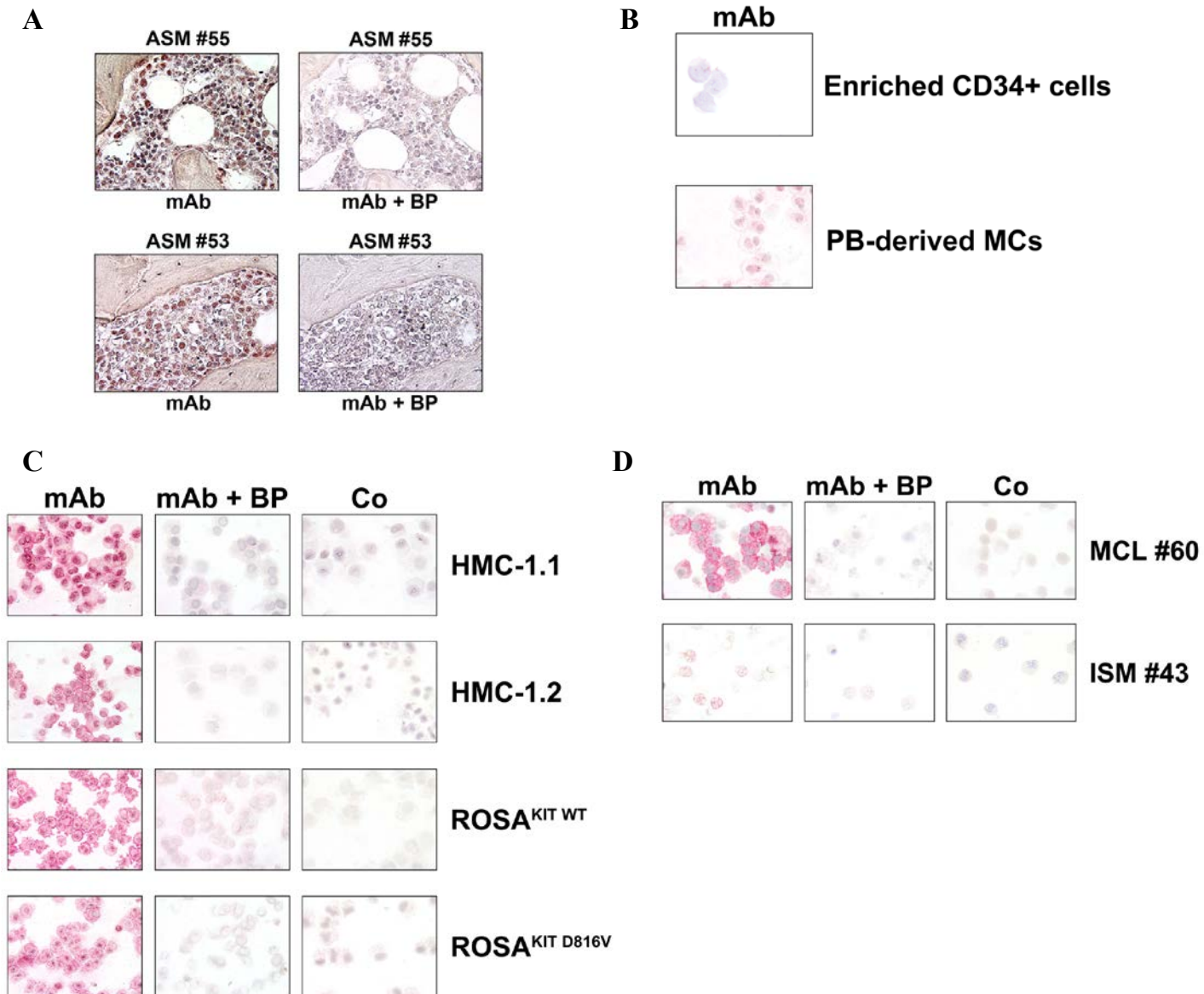
Gene	Sequence
BRD4-fwd	5'-GCCCCGCAAGCTCCAGGATGT-3'
BRD4-rev	5'-CCTCAGGCTCGTCCGGCATC-3'
MYC-fwd	5'-TGCTCCATGAGGAGACACC-3'
MYC-rev	5'-CCTGCCTCTTTTCCACAGAA-3'
MCL-1-fwd	5'-GTGCAGCGCAACCACGAG-3'
MCL-1-rev	5'-CGATTTACATCGTCTTCGTTT-3'
BCL-2-fwd	5'-TTGACAGAGGATCATGCTGTACTT-3'
BCL-2-rev	5'-TCAGTCTACTTCCTCTGTGATGTTGT-3'
BCL-XL-fwd	5'-CTCCTCTCCCGACCTGTGAT-3'
BCL-XL-rev	5'-AAGATTCTGAAGGGAGAGAAAGAGA-3'
BIM-fwd	5'-TGTCTGACTCTGACTCTCTGACTGA-3'
BIM-rev	5'-GAAGGTTGCTTTGCCATTTGGTC-3'
ABL-fwd	5'-TGTATGATTTTGGCCAGTGGAG-3'
ABL-rev	5'-GCCTAAGACCCGGAGCTTTTCA-3'

Abbreviations: qPCR, quantitative polymerase chain reaction; BRD4, bromodomain-containing protein 4; fwd, forward; rev, reverse; MYC, myelocytomatosis oncogene; MCL-1, myeloid cell leukemia sequence 1; BCL-XL, B-cell lymphoma-extra-large; BCL-2, B-cell leukemia/lymphoma 2; ABL, Abelson murine leukemia virus oncogene homolog 1.

Supplementary Figures

Supplementary Figure S1

Effect of a BRD4-specific blocking-peptide



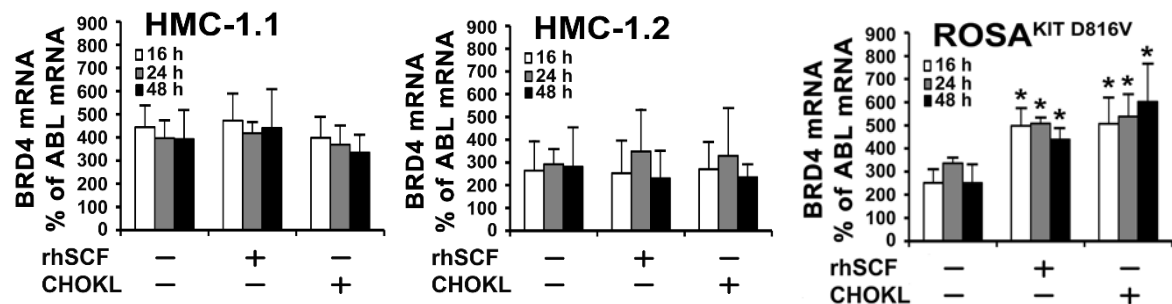
A: Bone marrow (BM) sections obtained from patients with ASM (patient #55 in upper panels and patient #53 in lower panels; # numbers refer to Table S2) were stained with a monoclonal antibody (mAb) against BRD4 by indirect immunohistochemistry. Before applied, the mAb was incubated in control buffer (left panels) or with a blocking peptide (mAb + BP) specific for BRD4 (right panels) for 1 hour. Stained sections were analysed by microscopy. Original magnification: x 60. B: Normal human CD34+ stem/progenitor cells (upper panel) and normal cultured, SCF-dependent, PB progenitor-derived mast cells (MCs) (lower panel) were stained with a

mAb against BRD4 on cytospin slides by indirect immunocytochemistry. Original magnification: x 100. C: HMC-1.1, HMC-1.2, ROSA^{KIT WT}, and ROSA^{KIT D816V} cells were spun on cytospin slides and stained with a mAb against BRD4 by indirect immunocytochemistry. Before applied, the mAb was incubated in control buffer (left panels) or with a blocking peptide (mAb + BP) specific for BRD4 (middle panels) for 1 hour. The antibody omission control (Co, right panels) is also shown. Stained slides were analysed by microscopy. Original magnification: x 100. D: Representative images of BRD4 staining in primary neoplastic MCs. BM derived MCs (>80% pure) from patients with MCL (patient #60, upper panel) or ISM (patient #43, lower panel) were spun on cytospin slides and stained with a mAb against BRD4 by indirect immunocytochemistry. Before applied, the mAb was incubated in control buffer (mAb, left panels) or with a blocking peptide specific for BRD4 (mAb + BP, middle panels) for 1 hour. The antibody omission control (Co, right panels) is also shown. Original magnification: x 100.

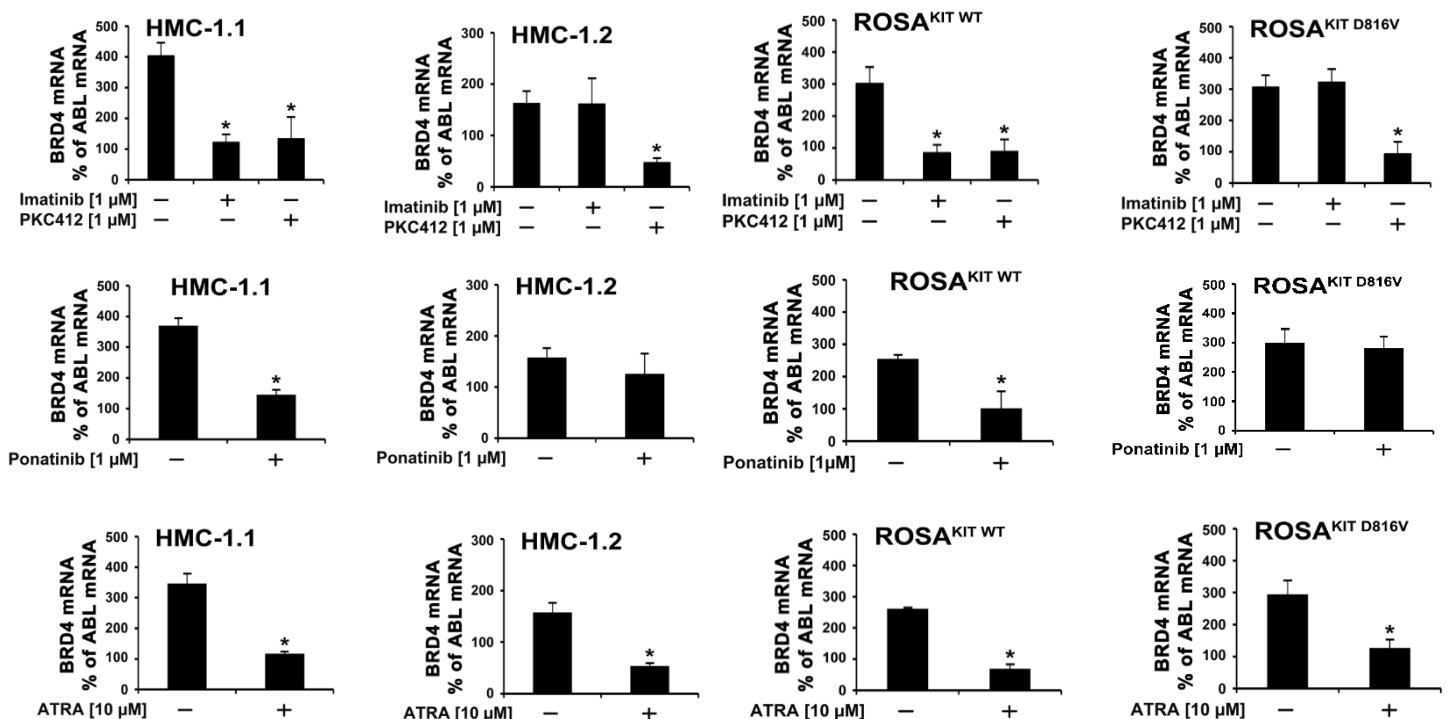
Supplementary Figure S2

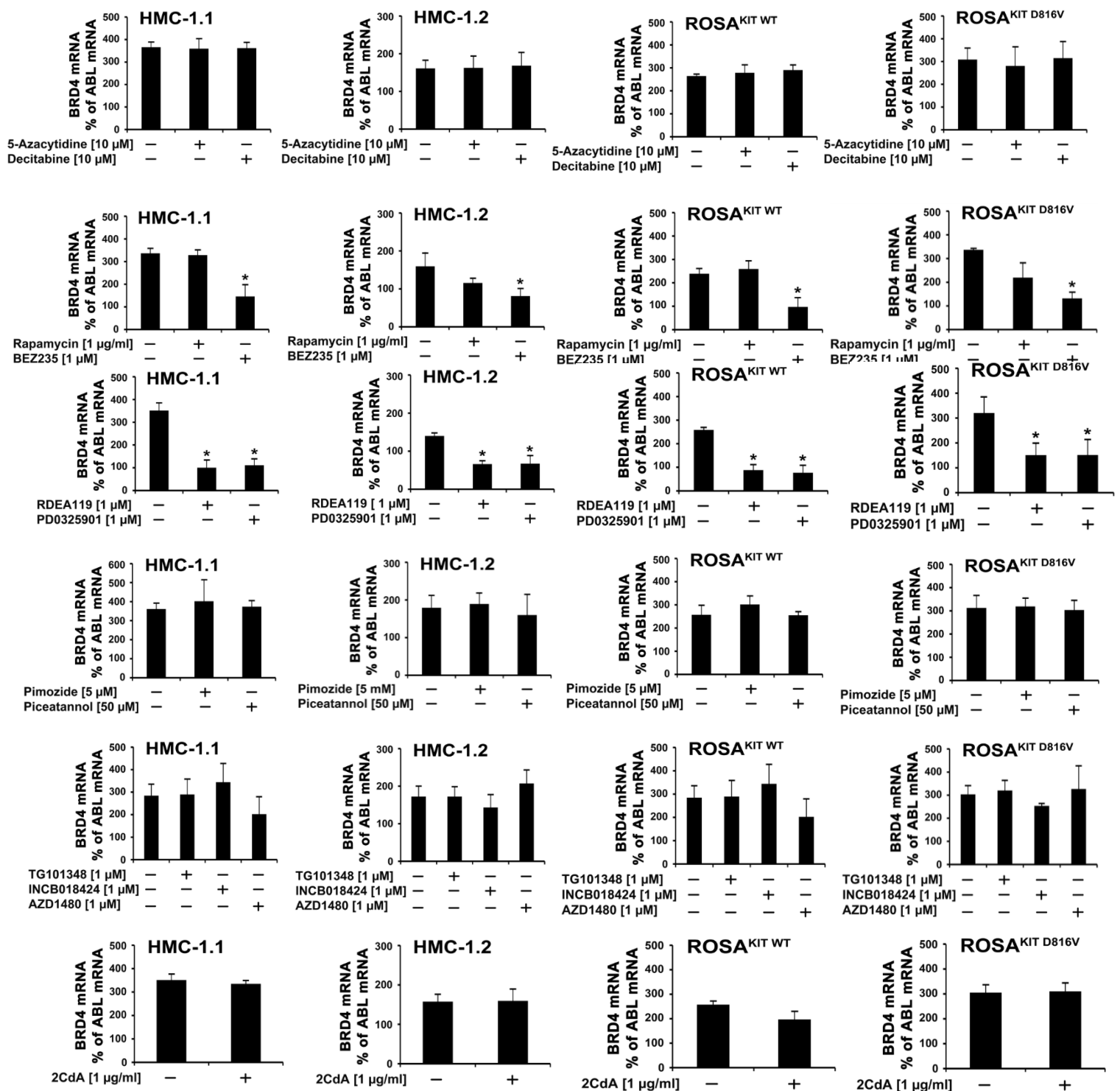
Effects of SCF and various drugs on expression of BRD4 mRNA in mast cells

A



B



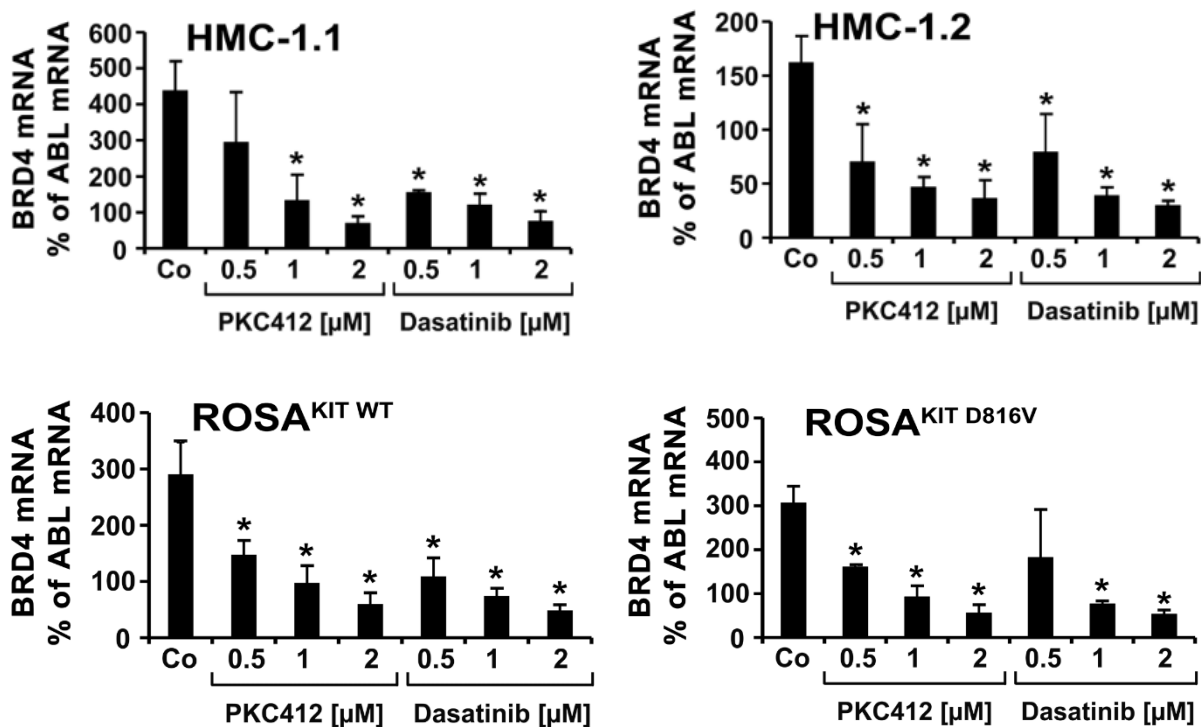


A: HMC-1.1 cells (left panel), HMC-1.2 cells (middle panel), and ROSA^{KIT D816V} cells (right panel) were incubated in control medium (-), rhSCF (200 ng/ml) or CHOKL (10% Chinese hamster ovary (CHO) supernatant containing murine SCF) at 37°C for 16 (open bars), 24 hours (gray bars) or 48 hours (black bars). Thereafter, cells were subjected to RNA isolation and qPCR. Results show BRD4 mRNA expression levels as percent of ABL mRNA levels, and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to control. B: HMC-1.1 cells,

HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (–) or medium containing imatinib (1 μM), PKC412 (1 μM), ponatinib (1 μM), ATRA (10 μM), the demethylating agents 5-azacytidine and decitabine (10 μM each), the mTOR blocker rapamycin (1 μg/ml), BEZ235 (1 μM), the MEK1/2 inhibitors RDEA119 and PD0325901 (1 μM each), the STAT5 blockers pimozide (5 μM) and piceatannol (50 μM), the JAK2 inhibitors TG101348, INCB018424 and AZD1480 (1 μM each) and 2CdA (cladribine) (1 μg/ml) at 37°C for 12 hours. Then, RNA was isolated and BRD4 mRNA levels were quantified by qPCR. Results show BRD4 mRNA levels as percent of ABL mRNA levels and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to control.

Supplementary Figure S3

PKC412 and dasatinib dose-dependently downregulate the expression of BRD4 in HMC-1 cells and ROSA cells

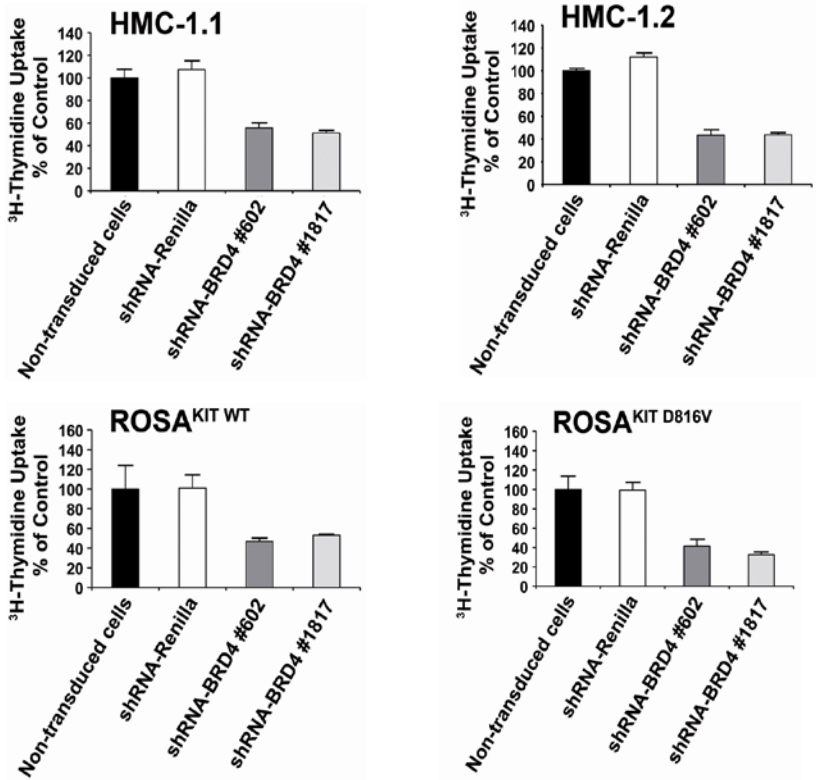


HMC-1.1 cells (upper left panel), HMC-1.2 cells (upper right panel), ROSA^{KIT WT} cells (lower left panel) and ROSA^{KIT D816V} cells (lower right panel) were incubated in control medium (Co) or in medium containing 0.5, 1 or 2 μM of PKC412 or dasatinib (as indicated) at 37°C for 12 hours. Thereafter, RNA was isolated and BRD4 mRNA levels were quantified by qPCR. Results show BRD4 mRNA levels as percent of ABL mRNA levels and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates: p<0.05.

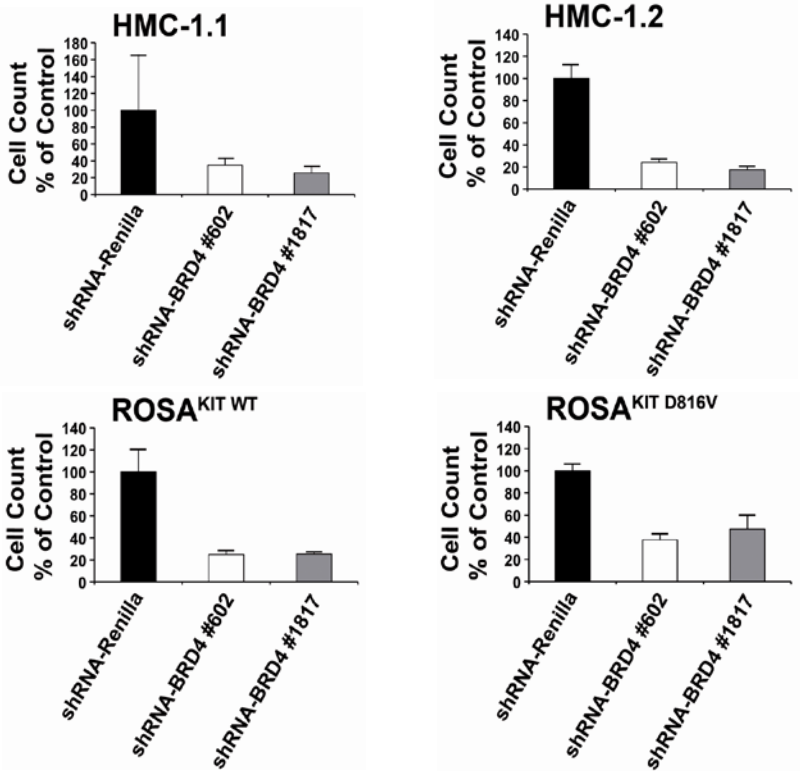
Supplementary Figure S4

Effects of BRD4 knock-down on the proliferation of human mast cell lines

A

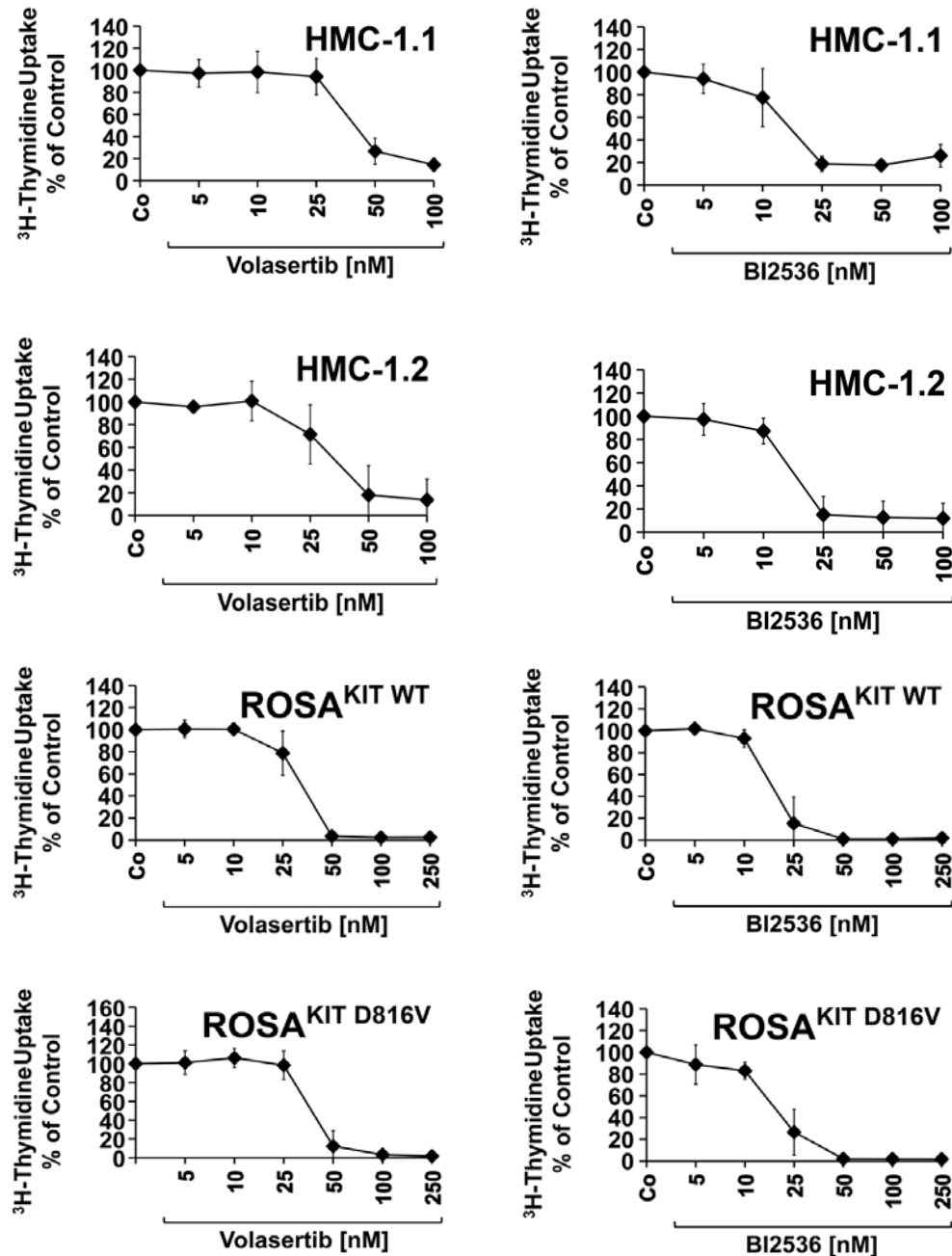


B



Effects of Volasertib (BI6727) and BI2536 on the proliferation of HMC-1 cells and ROSA cells

C

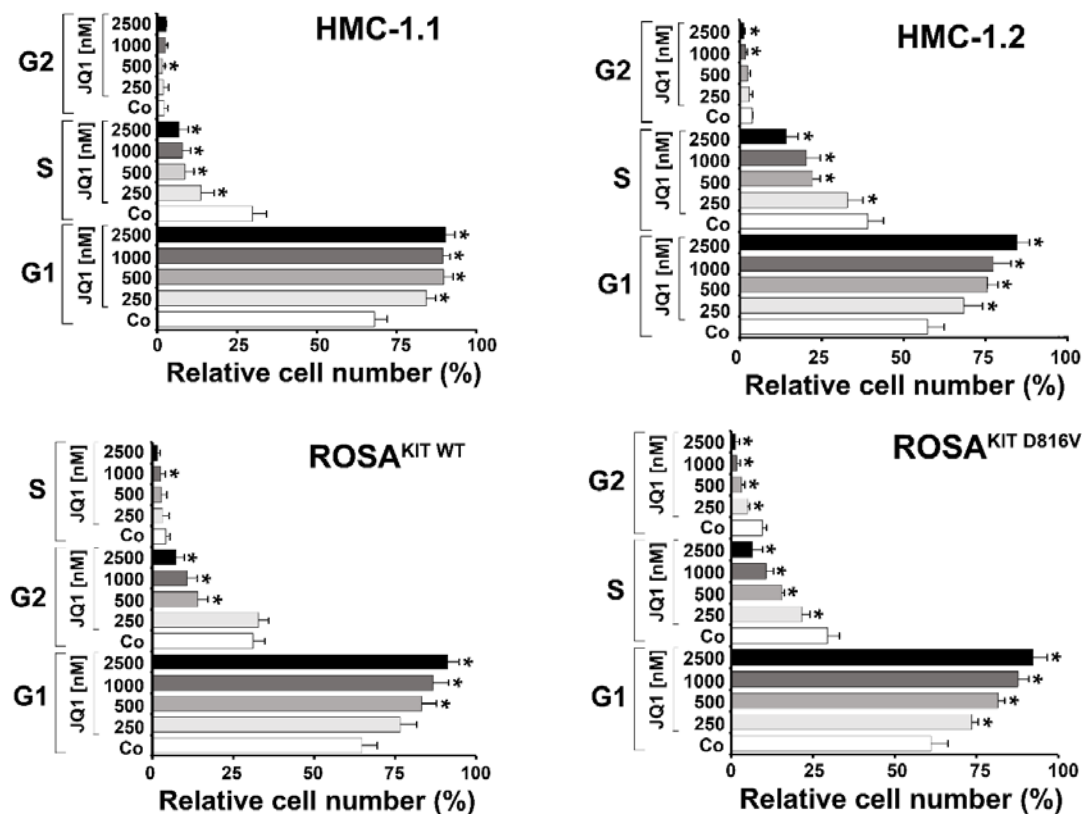


A: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were transduced with a control shRNA targeting Renilla luciferase (shRNA-Renilla) (black bars) or with two different shRNA constructs targeting BRD4 (shRNA-BRD4 #602 and #1817) as indicated. GFP⁺ cells and non-transduced control cells were sorted and maintained at 37°C for 24 hours. Then, ³H-thymidine uptake was measured. Results

are expressed as percent of control and represent the mean±S.D. of triplicates. B: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were transduced with a control shRNA (shRNA-Renilla) or with two different shRNA constructs targeting BRD4 (shRNA-BRD4 #602 and #1817). GFP⁺ cells were sorted and maintained at 37°C. Cell counts were assessed on day 7. Results are expressed as percent of shRNA control and represent the mean±S.D. of triplicates. C: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in the absence (Co) or presence of increasing concentrations of either Volasertib (left panels) or BI2536 (right panels) at 37°C for 48 hours. Then, ³H-thymidine uptake was measured. Results are expressed as percent of control (Co) and represent the mean±S.D. of 3 independent experiments.

Supplementary Figure S5

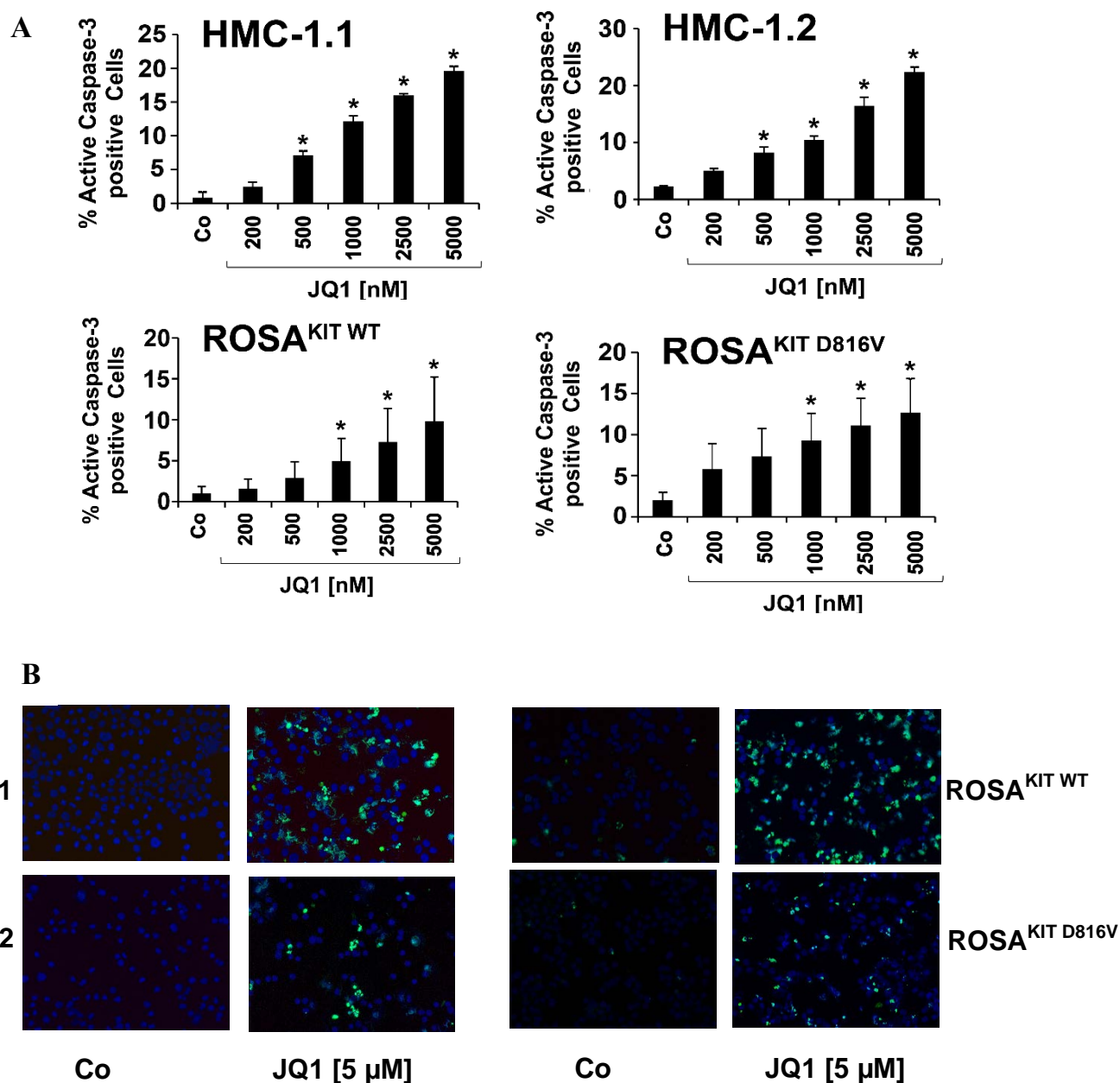
JQ1 induces cell cycle arrest in HMC-1 cells and ROSA cells



HMC-1.1 cells (upper left panel), HMC-1.2 cells (upper right panel), ROSA^{KIT WT} cells (lower left panel) and ROSA^{KIT D816V} cells (lower right panel) were incubated in control medium (Co) (open bars) or medium containing various concentrations of JQ1 (250-2,500 nM; grey and black bars) at 37°C for 24 hours. Thereafter, cells were lysed and stained with propidium iodide (PI). Results show the percentage of cells in each cell cycle phase (S, G2, G1) and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to control.

Supplementary Figure S6

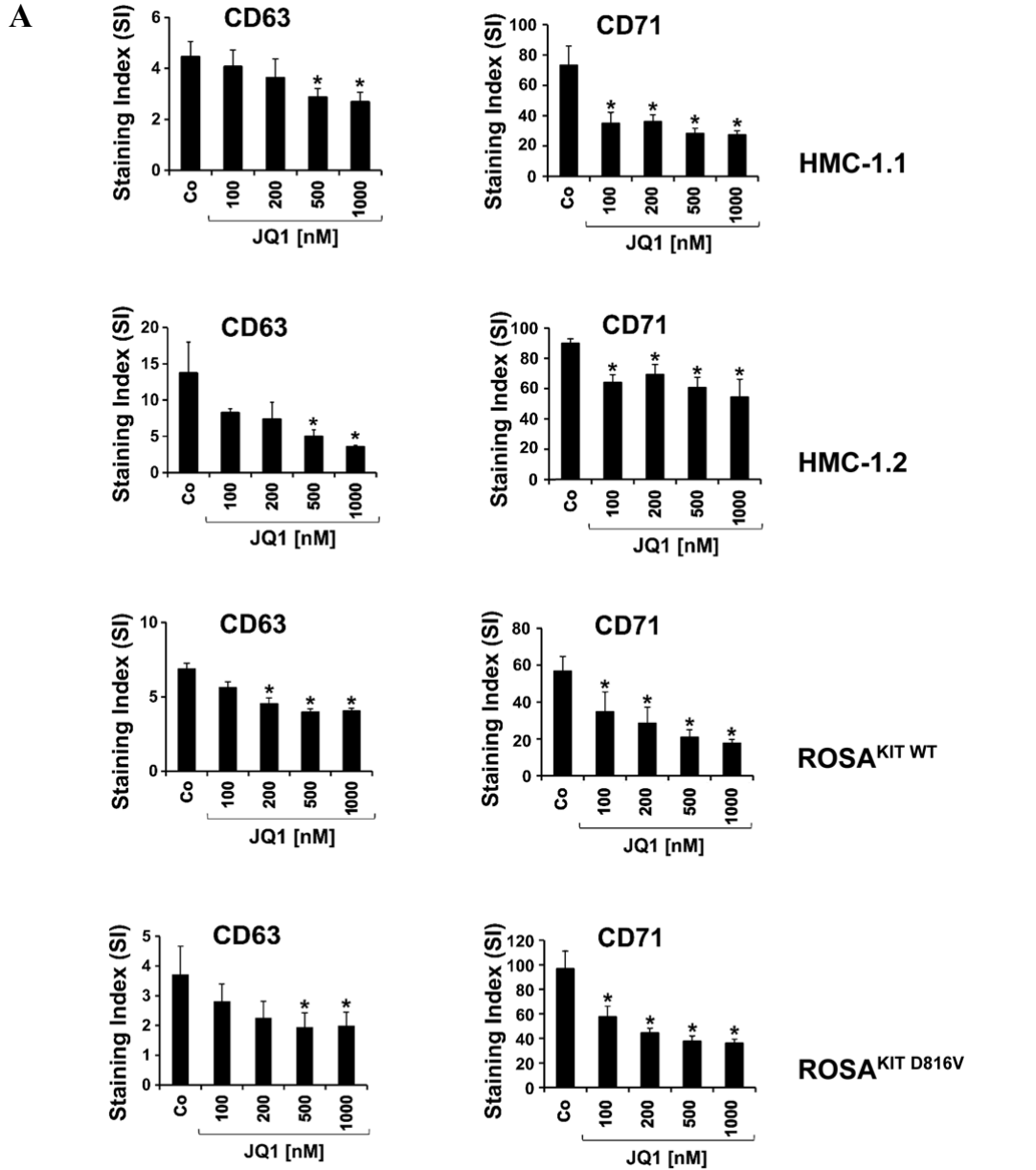
Effects of JQ1 on the viability of human mast cell lines



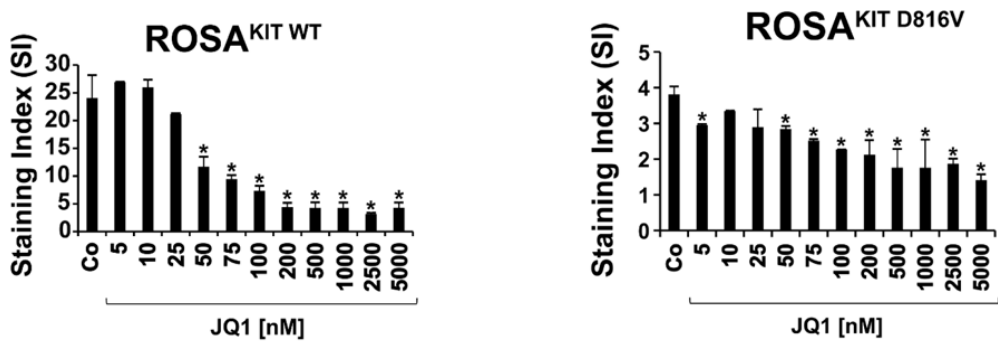
A: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (Co) or in various concentrations of JQ1 for 48 hours. Then, cells were fixed, permeabilized by methanol, incubated with an antibody against active caspase-3 and analysed by flow cytometry. Results are expressed as percent of caspase-3 positive cells and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates $p < 0.05$ compared to control (Co). B: HMC-1 and ROSA cells were incubated with 5 μM JQ1 for 48 hours and then examined for the presence of apoptotic cells by a TUNEL assay. Original magnification: x 20.

Supplementary Figure S7

Effects of JQ1 on expression of CD63 and CD71 on HMC-1 and ROSA cells



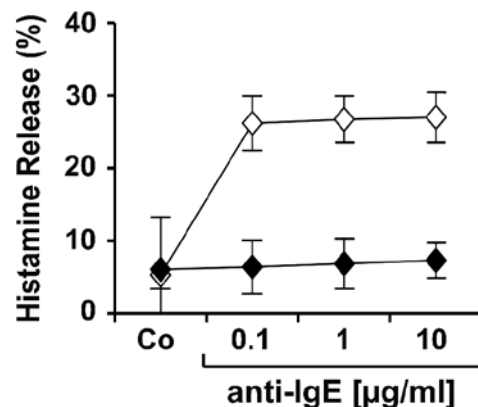
B



A: HMC-1.1, HMC-1.2, ROSA^{KIT WT} and ROSA^{KIT D816V} cells were incubated in control medium (Co) or various concentrations of JQ1 at 37°C for 48 hours. Then, expression of CD63 (left panels) and CD71 (right panels) was analyzed by flow cytometry. Results are expressed as staining index (SI = mean fluorescence intensity values of antibody relative to an isotype-control) and represent the mean±S.D. of 3 independent experiments. B: FcεRI expression on ROSA^{KIT WT} cells (left panel) and ROSA^{KIT D816V} cells (right panel) after incubation with control medium (Co) or various concentrations of JQ1 at 37°C for 48 hours. Results are expressed as SI and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to untreated cells.

Supplementary Figure S8

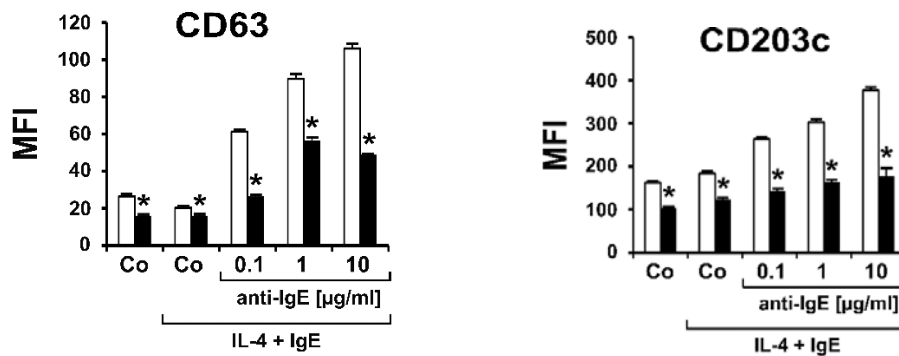
Effects of JQ1 on histamine release in ROSA^{KIT WT} cells



ROSA^{KIT WT} cells were incubated with IL-4 and IgE for 80 hours. Then, control medium (◇-◇) or JQ1 (200 nM) (◆-◆) was added for 16 hours. Thereafter, cells were washed and exposed to various concentrations of anti-IgE (0.1, 1, or 10 µg/ml) or control buffer (Co) for 30 minutes. Cells were then centrifuged and cell-free supernatants and cell suspensions were recovered and subjected to histamine measurements by RIA. Results are expressed as percentage of total histamine and represent the mean±S.D. of 3 experiments.

Supplementary Figure S9

JQ1 inhibits IgE-mediated upregulation of CD63 and CD203c in ROSA cells

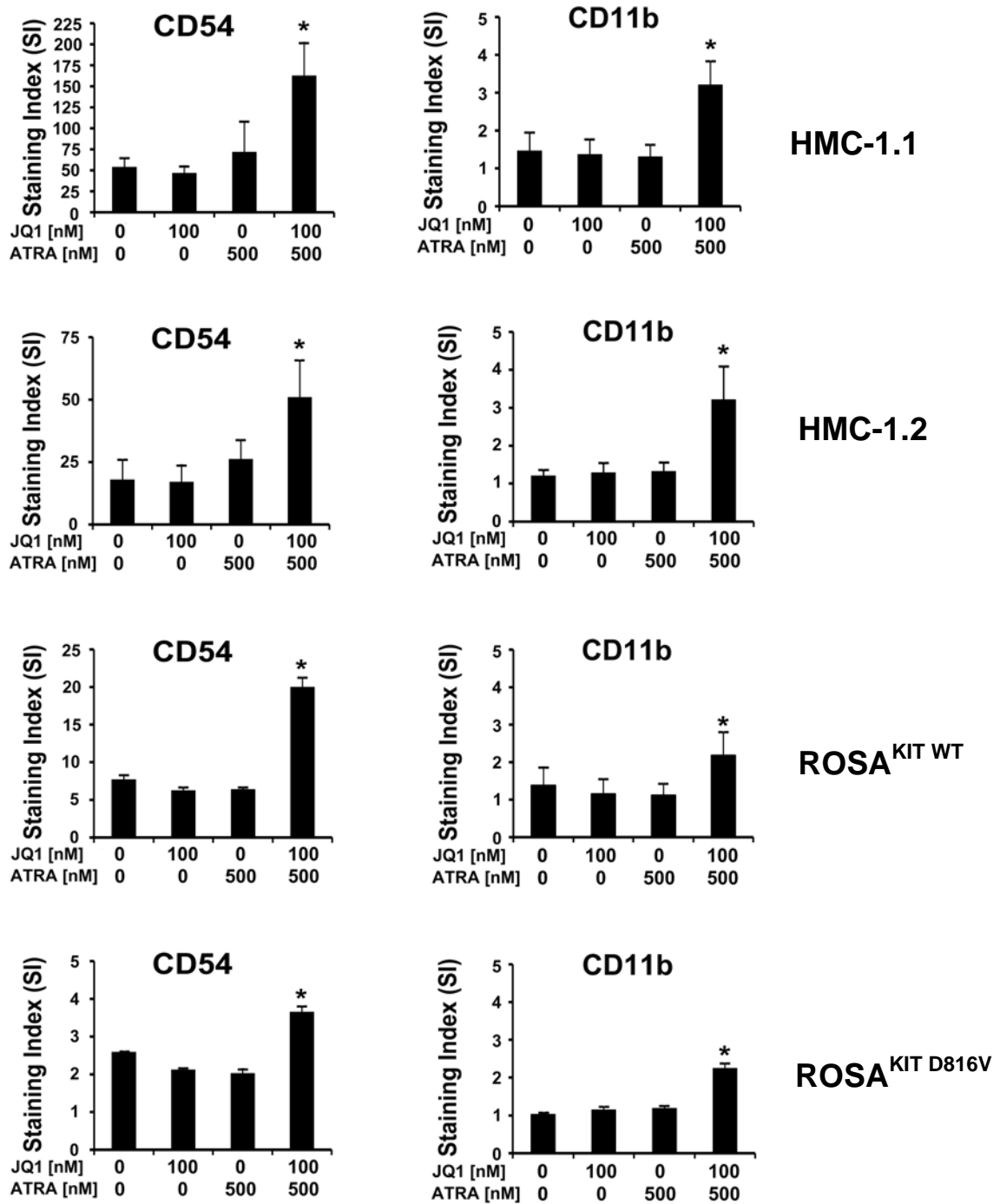


ROSA^{KIT^{WT}} cells were primed with IL-4 (10 ng/ml) and IgE for 80 hours and then incubated with control medium (Co) or medium containing JQ1 (200 nM) at 37°C for 16 hours. Thereafter, cells were washed and incubated with 3 different concentrations of anti-IgE (0.1, 1 and 10 µg/ml) at 37°C for 30 minutes. Mean fluorescence intensities (MFI), produced by staining with antibodies against CD63 and CD203c, were measured on a FACSCalibur. Results represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05.

Supplementary Figure S10

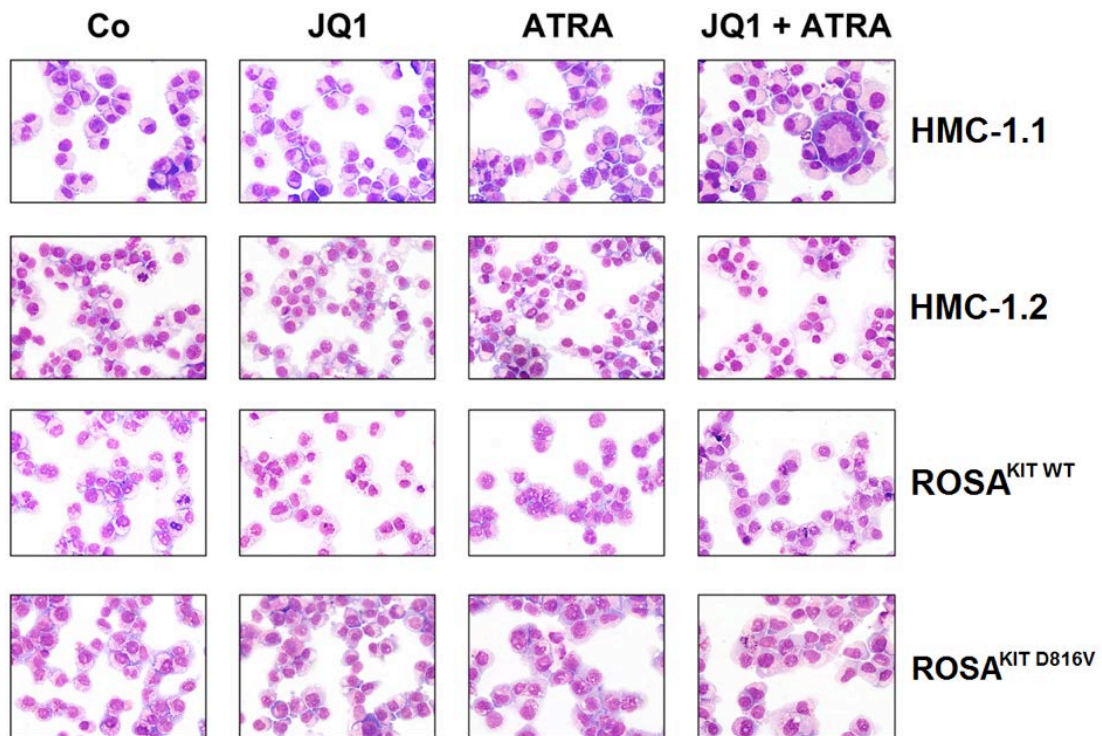
Effects of JQ1 on the expression of CD54 and CD11b on human mast cell lines

A



Effects of JQ1 and ATRA on maturation of HMC-1 cells and ROSA cells

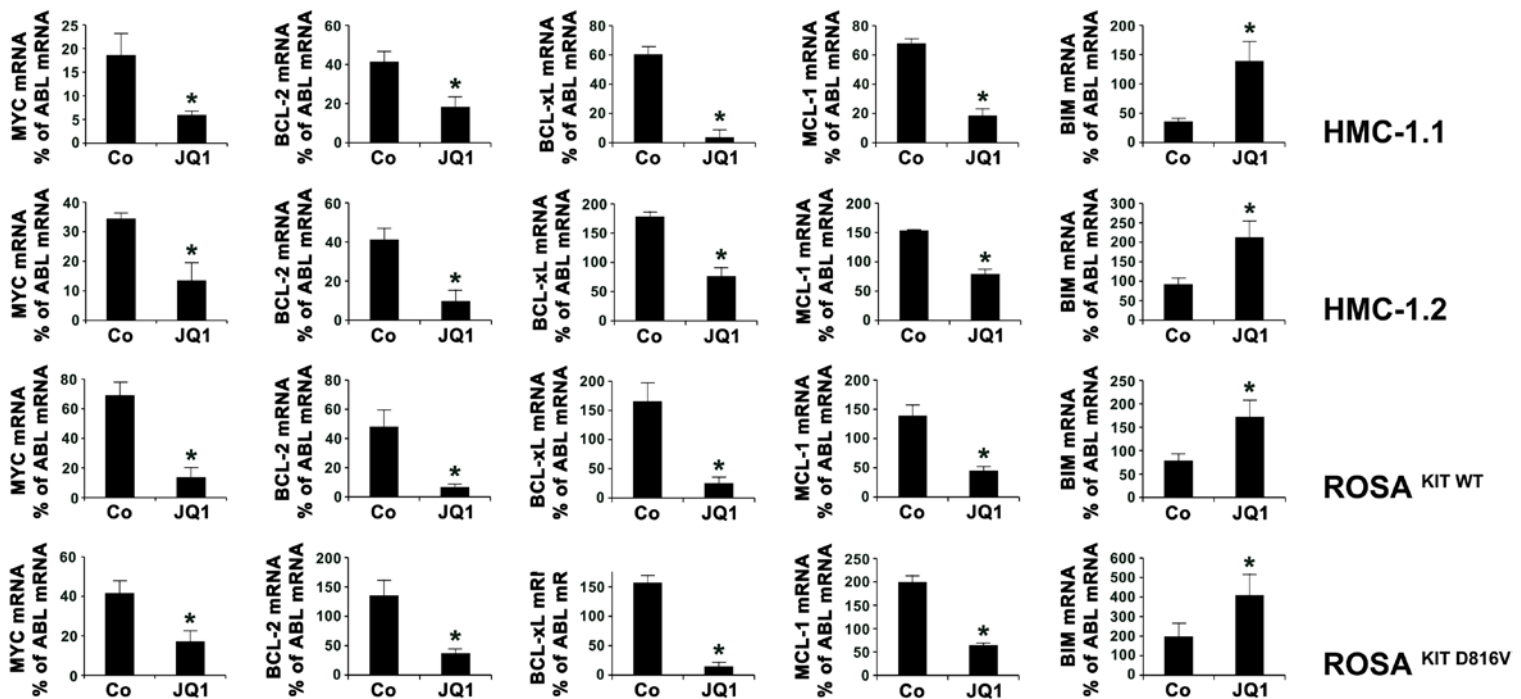
B



A: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (0) or in medium containing ATRA and JQ1 in various concentrations alone or in combination (as indicated) at 37°C for 48 hours. Then, expression of CD11b (right panels) and CD54 (left panels) was analyzed by flow cytometry. Results are expressed as staining index (SI = mean fluorescence intensity values of tested antibody relative to an isotype-control) obtained with viable cells and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates $p < 0.05$ compared to untreated cells. B: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (Co) or in medium containing JQ1 (100 nM) and ATRA (500 nM) alone or in combination (JQ1 + ATRA) at 37°C for 48 hours. Thereafter, cells were washed, spun on cytopsin slides and stained with Wright-Giemsa solution. Original magnification: x 60.

Supplementary Figure S11

Effects of JQ1 on expression of MYC and BCL-2-family members in HMC-1 cells and ROSA cells

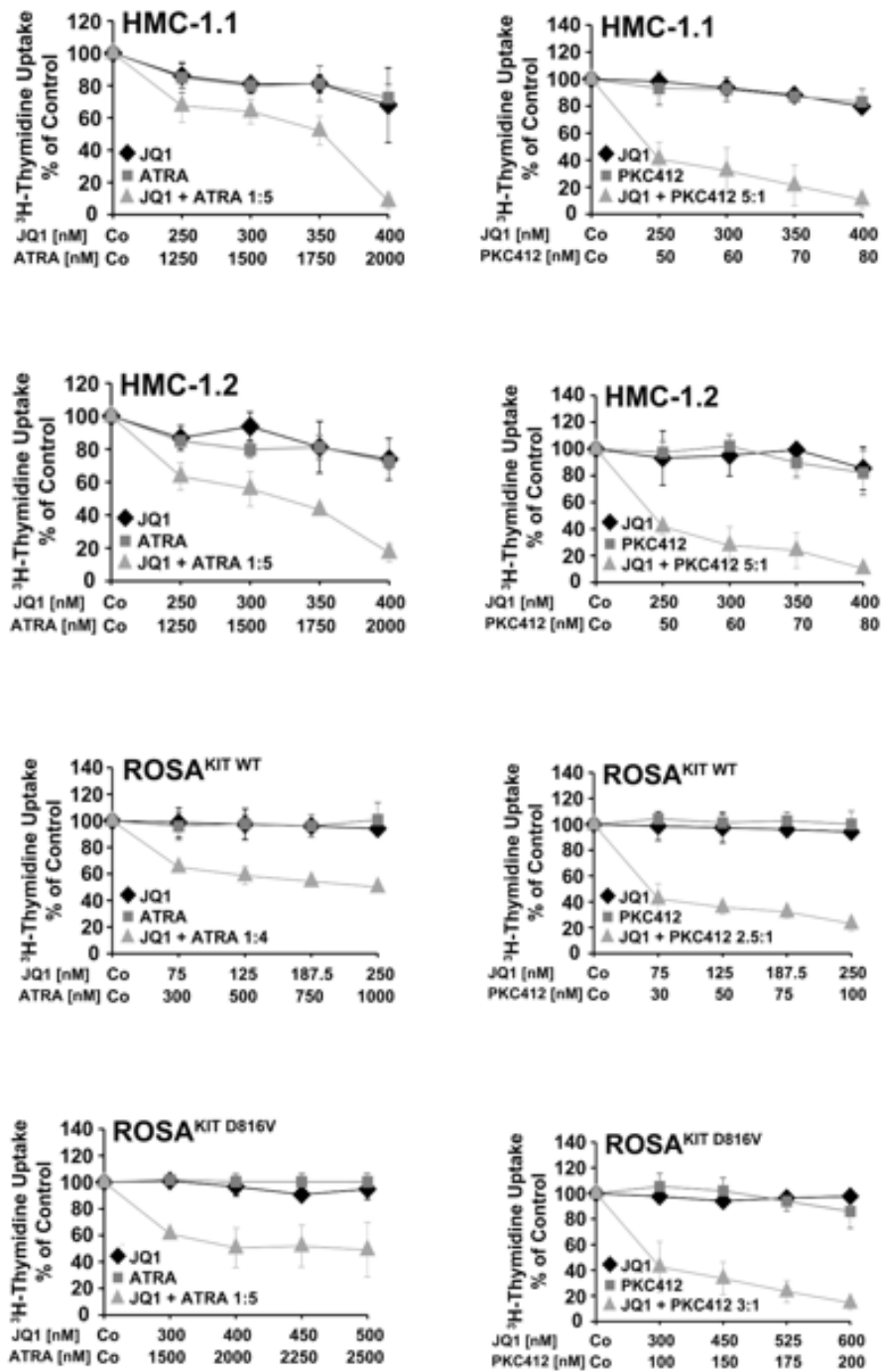


HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (Co) or medium containing JQ1 (2,500 nM) at 37°C for 24 hours. Then, RNA was isolated and BCL-2-, MCL-1-, BCL-xL-, BIM-, and MYC-mRNA levels were quantified by qPCR as described in the main document. Results show transcript expression levels as percent of ABL mRNA expression and represent the mean±S.D of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to control.

Supplementary Figure S12

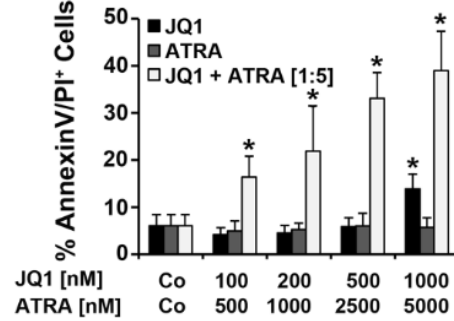
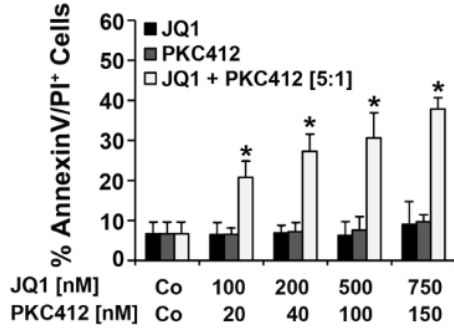
JQ1 synergizes with all-trans retinoic acid (ATRA) and PKC412 (midostaurin) in inducing apoptosis in HMC-1 cells and ROSA cells

A

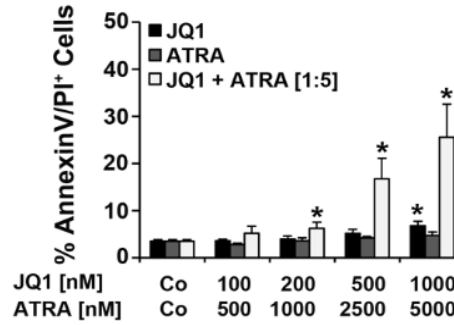
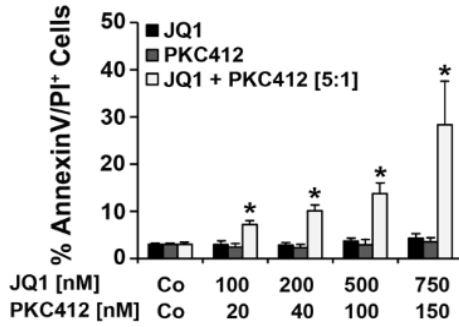


B

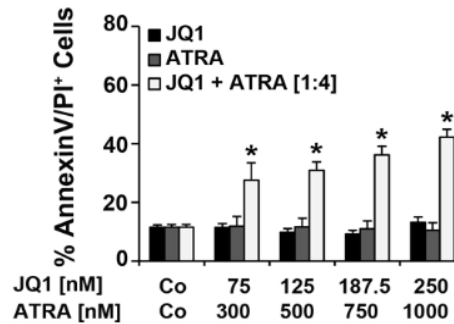
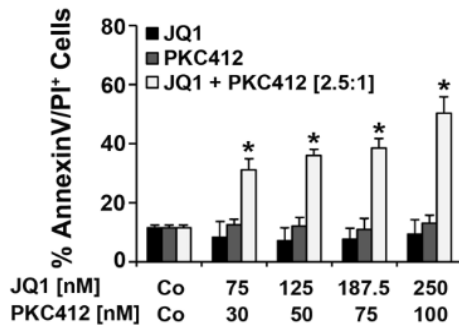
HMC-1.1



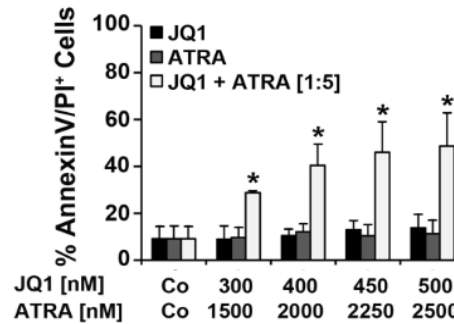
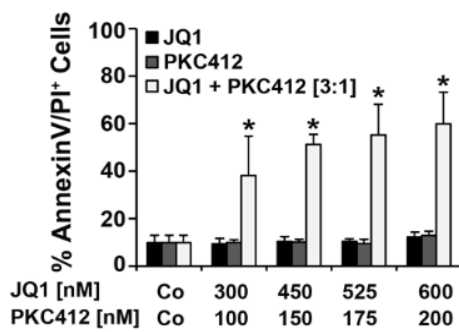
HMC-1.2



ROSA^{KIT WT}

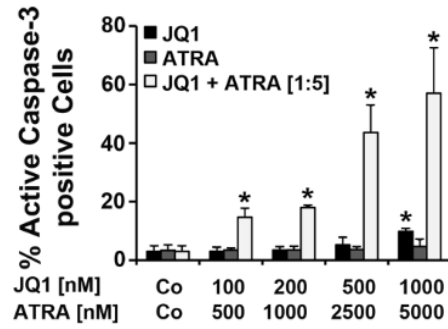
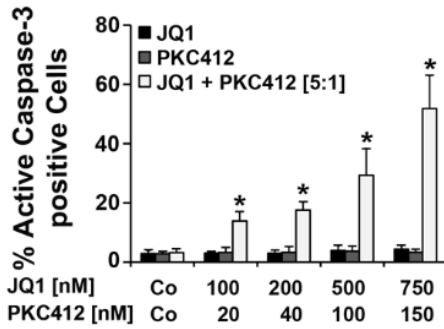


ROSA^{KIT D816V}

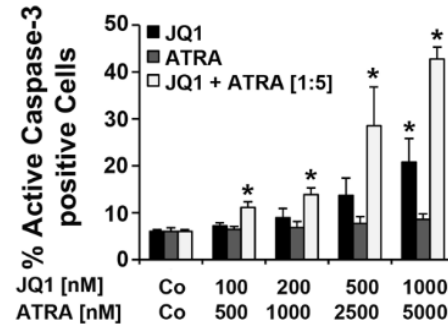
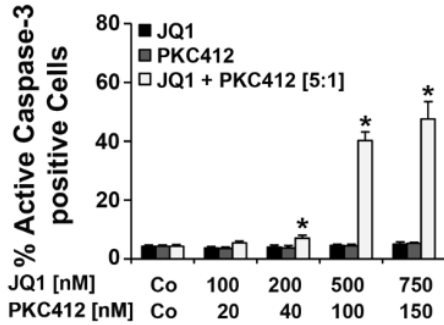


C

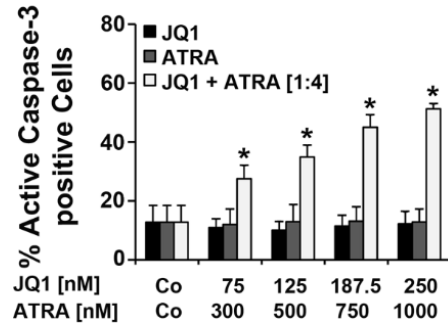
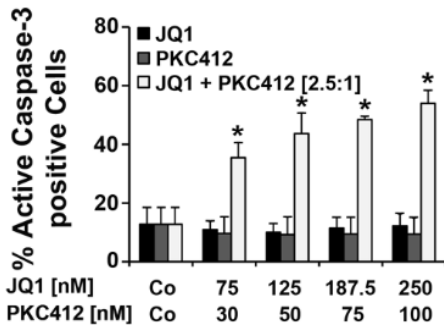
HMC-1.1



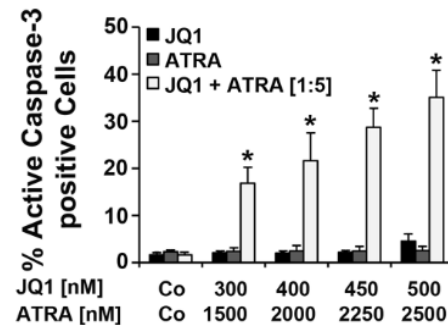
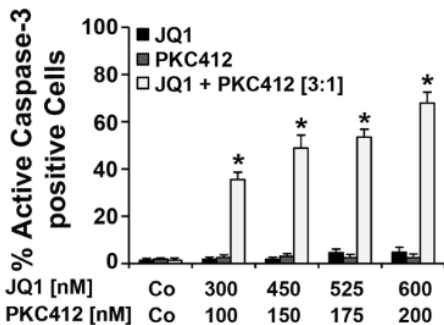
HMC-1.2



ROSA^{KIT WT}



ROSA^{KIT D816V}



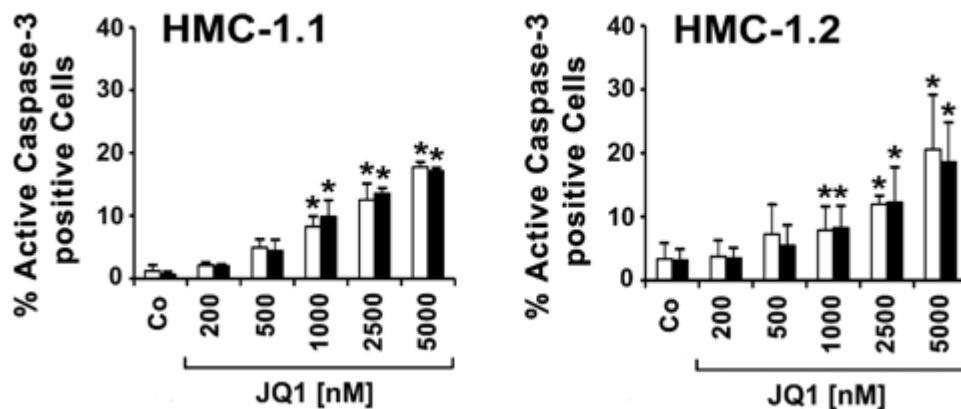
A: HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells were incubated in control medium (Co) or in medium containing various concentrations of JQ1, ATRA, or PKC412 alone or in combinations (as indicated) at 37°C for 48 hours. Then, cells were analyzed for uptake of ³H-thymidine. Results are expressed as percent of control (Co) and represent the mean±S.D. of 3 independent experiments. B, C: HMC-1 cells (upper images) and ROSA cells (lower images) were incubated in control

medium (Co) or various concentrations of JQ1 (black bars), PKC412 (dark gray bars, left images) or ATRA (dark gray bars, right images) alone or in combination (open bars) for 48 hours. Then, cells were stained for AnnexinV/Propidium iodide (B) or active caspase-3 expression (C) by flow cytometry. Results represent the mean±S.D. of 3 independent experiments. Asterisk (*): $p < 0.05$ compared to control (Co).

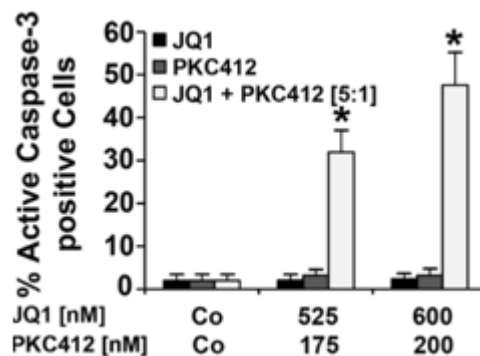
Supplementary Figure S13

Effects of stem cell factor (SCF) on responsiveness of neoplastic MCs to JQ1

A



B



A: Effects of SCF on JQ1-mediated apoptosis in HMC-1 cells. HMC-1.1 cells (left panel) and HMC-1.2 cells (right panel) were incubated in the absence (open bars) or presence of rhSCF (200 ng/ml) (black bars) at 37°C for 24 hours. Then, cells were washed and incubated in control medium (Co) or in various concentrations of JQ1 (200-5,000 nM) at 37°C for 48 hours. Thereafter, the percentage of active caspase-3 positive cells was measured. Results represent the mean±S.D. of 3 independent experiments. Asterisk (*): $p < 0.05$ compared to Co. B: ROSA^{KIT}D816V cells were incubation with rhSCF (200 ng/ml) and treated with JQ1 (black bars) or PKC412 (dark gray bars) alone or in combination (light gray bars) at 37°C for 48 hours. Then, the percentage of active caspase-3 positive cells was analysed by flow cytometry. Results represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates $p < 0.05$.

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UNPUBLISHED SUPPLEMENTAL DATA

SUPPLEMENTAL MATERIALS AND METHODS

1. Chemical reagents and drugs

The proteasome inhibitors Bortezomib and MG132 were purchased from ChemieTek and Selleckchem (Houston, TX, USA). They were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich).

2. Canine cell lines

The canine MCL cell line NI-1 (Hadzijušufovic *et al.* 2012) was maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 1% amphotericin and 1% antibiotics in 5% CO₂ at 37°C. The canine mastocytoma cell line C2 (DeVinney & Gold 1990) was kindly provided by Dr. W. Gold (University of California, San Francisco, CA, USA). C2 cells were cultured in IMDM supplemented with 10% FCS, α -thioglycerol, L-glutamine, and antibiotics in 5% CO₂ at 37°C.

3. Methods

3.1. Evaluation of the expression of STAT proteins by flow cytometry

In selected experiments, intracellular staining for p-STAT3 and p-STAT5 was conducted after 4, 16 and 48 hours of incubation of ROSA^{KIT WT} in control medium or in medium containing different concentrations of JQ1. After incubation, cells were washed with PBS then fixed with paraformaldehyde 2% for 15 minutes at RT. Thereafter, cells were centrifuged and the cell pellet was resuspended in cool methanol at -20°C for at least 20 minutes. Cells then were washed with PBS containing 0.1% of bovine serum albumin (BSA) and the Alexa Fluor conjugated antibodies against p-STAT 3 (Alexa Fluor[®] 647 mouse anti-STAT3 (pY705), clone 4/p-STAT3, BD Biosciences) or against p-STAT 5 (Alexa Fluor[®] 647 mouse anti-STAT5 (pY694), clone 47, BD Biosciences) or the corresponding isotype control (Alexa Fluor[®] 647 mouse IgG1 κ or IgG2a κ) were added. Cells were then incubated in the dark for 30 minutes at RT. After one washing step with PBS 0.1% BSA, cell pellet was resuspended in about 30 μ L of PBS 0.1% BSA and fluorescence was analyzed with a

FACSCalibur (BD Biosciences).

3.2. Western Blotting

Prior to Western Blotting, HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells, and ROSA^{KIT D816V} cells (for each, 10⁶ cells/ml) were incubated in control medium or medium containing different concentrations of JQ1, (1-5 μM) at 37°C for 24 hours. Thereafter, cells were harvested and washed once with PBS at 4°C and resuspended in RIPA lysis buffer (1 mL/10⁸ cells) consisting of 50 mmol/L of Tris (pH 7.5), 150 mmol/L of sodium chloride (NaCl), 0.1% (vol/vol) NP-40, 0.1% (wt/vol) sodium deoxycholate, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 1 mmol/L of sodium fluoride, 1 mmol/L of sodium orthovanadate and one protease inhibitor cocktail table (Complete Mini, Roche, Indianapolis, IN, USA). Lysates were then incubated on ice under vigorous saking for 30 minutes, and centrifuged for 10 minutes (20000 g) at 4°C to remove insoluble particles.

For Western Blot analysis, protein amount was measured with the BC Assay protein Kit (Interchim, Mountluçon, France) using bovine serum albumin (BSA) Standard (Interchim) and the calculated loading amounts (50-100 μg) were heated with blue loading buffer (SB1X containing 25 mmol/L of Tris 1 mol/L (PH 6.8), 0.8% of SDS 10%, 3.5% of glycerol and 0.0005% of bromophenol blue (all from Sigma–Aldrich) and complemented with the reducing agent Dithiothreitol (DTT) 10 mmol/L) at 95°C for 10 minutes. Afterwards, the samples were loaded and separated under reducing conditions by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane (Amersham™ Hybond™ –ECL 0.45 μ, GE Healthcare, Waukesha, WI, USA) in buffer containing 25 mM of Tris, 192 mM of glycine, and 20% (vol/vol) of methanol at 4°C. The membrane was blocked for 1 hour in 5% of bovine serum albumin (BSA) (Sigma-Aldrich) or 5% (wt/vol) notfat dry milk powder in Tris-buffered saline (20 mM of Tris-hydrochloric acid and 150 m mM of NaCl, PH 7.5). The membrane was then incubated sequentially with the primary antibody overnight at 4°C with gentle shaking and the secondary horseradish peroxidase (HRP)-coupled antibody for 1 hour at RT with gentle shaking. Both primary and secondary antibodies were diluted in 5 % BST w/v, 1X TBS, and 0.1% Tween-20 Tris-buffered saline Amersham, Piscataway, NJ) for 1 hour. HRP activity was

detected by using HRP substrates (Amersham[™] ECL[™] Western Blotting Analysis System, GE Healthcare) and chemiluminescence film (Amersham Hyperfilm[™] ECL, GE Healthcare).

The following primary Abs were used: a polyclonal Ab against p-KIT (Tyr719, Cell Signaling Technology, Danvers, MA, USA), a polyclonal Ab against KIT, a polyclonal Ab against p-STAT5 (Tyr694, Cell Signaling Technology), a polyclonal Ab against STAT5 (Cell Signaling Technology), a polyclonal Ab against p-p44/42 MAP (ERK1/2) (Thr202/Tyr204, Cell Signaling Technology), a polyclonal Ab against p-Akt (Ser473, Cell Signaling Technology), a polyclonal Ab against AKT (Cell Signaling Technology), a mAb against total p44/42 MAP (ERK1/2) (Cell Signalling Technology), a mAb against p-STAT3 (Millipore, Billerica, MA, USA), a mAb against STAT3 (Millipore), a mAb against BTK (Cell Signaling Technology), a polyclonal Ab against p-BTK (Tyr223, Cell Signaling Technology), a mAb against c-MYC (Cell Signalling Technology), a mAb against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GAPDH 6C5, Santa Cruz Biotechnology, Dallas, Texas, USA) and a polyclonal AB against MC tryptase (Santa Cruz Biotechnology).

The secondary Abs used were: a polyclonal goat anti-rabbit IgG conjugated to HRP (Dako, Glostrup, Denmark), a donkey anti-goat IgG conjugated to HRP (Santa Cruz Biotechnology), or a polyclonal goat anti-mouse IgG conjugated to HRP (Rockland H & L, Rockland, MA, USA).

For densitometrical analysis of the Western Blots for phosphorylated proteins, the blots were scanned and then the expression of proteins was calculated and normalized against GAPDH for every cell line.

3.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

In selected experiments, using Trizol® (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from ROSA^{KIT^{WT}} cells preincubated with control medium or medium containing 200 nM JQ1 for 16 hours at 37°C. The cDNA was then synthesized from 1 µg of total RNA extract using Bio-Rad Kit (Hercules, CA, USA) according to the manufacturer's instructions. The cDNA was used for the template of PCR with Crimson Taq DNA polymerase (New England BioLabs, Ipswich, Massachusetts). The polymerase chain reaction (PCR) conditions were: denaturation 30 s (94°C); annealing 30 s (55°C); extension, 1 min (72°C); 35 cycles. Equal loading was confirmed by

Table XI : Sequences of oligonucleotide primers used for RT-PCR.

Gene	Sequence
FcεRIα –fwd	5'-CTATGAGTCTGAGCCCCTCAA-3'
FcεRIα –rev	5'-TTCTCACGCGGAGCTTTTATT-3'
FcεRIβ –fwd	5'-AGAGCAAATCTTGCTCTCCCAC-3'
FcεRIβ –rev	5'-TTCAAATGCAGGCACACTGG-3'
FcεRI γ –fwd	5'-AGGGCCGCTGCTTGTTTC-3'
FcεRI γ –rev	5'-TCCAGCAGTGGTCTTGCTCTT -3'
HRPT-fwd	5'-ATGGACAGGACTGAACGTCTTGC-3'
HRPT-rev	5'-GACACAAACATGATTCAAATCCCTGA-3'

determining HRPT (hypoxanthine guanine phosphoribosyltransferase) mRNA levels using primers depicted in Table XI. The PCR conditions were: denaturation 15 s (95°C); annealing and extension 1 min (60°C).

Electrophoresis of the PCR products on 1.5% agarose gel (Euromedex, Souffelweyersheim, France) was then visualized by ethidium bromide (Electran[®], BDH Prolabo, VWR International, England) and scanned. The FcεRI mRNA expression levels were quantified by densitometry using ImageJ software (<http://rsb.info.nih.gov/gate2.inist.fr/ij>) and were expressed after correcting for HRPT mRNA expression levels.

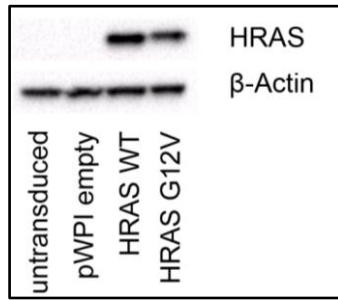


Figure S1: Western Blot (WB) analysis of ROSA^{KIT^{WT}} cells lentivirally transduced with pWPI.

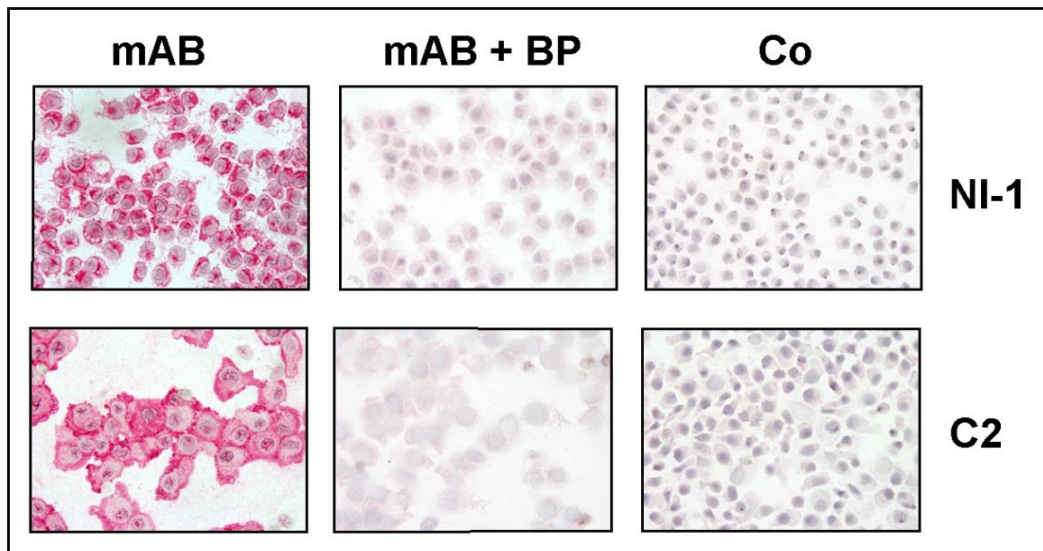


Figure S2: Expression of BRD4 in neoplastic canine MC lines. The canine MCL cell line NI-1 (upper panels) and the canine mastocytoma cell line C2 (lower panels) were spun on cytospin slides and stained with a mAb against BRD4 (left panel) or antibody omission control (Co, right panels) by indirect immunocytochemistry. Before applied, the mAb was incubated in control buffer (left panels) or with a blocking peptide (mAb + BP) specific for BRD4 (middle panels) for 1 hour. Stained slides were analysed by microscopy, and images were taken and adjusted as described. Original magnification: x 100.

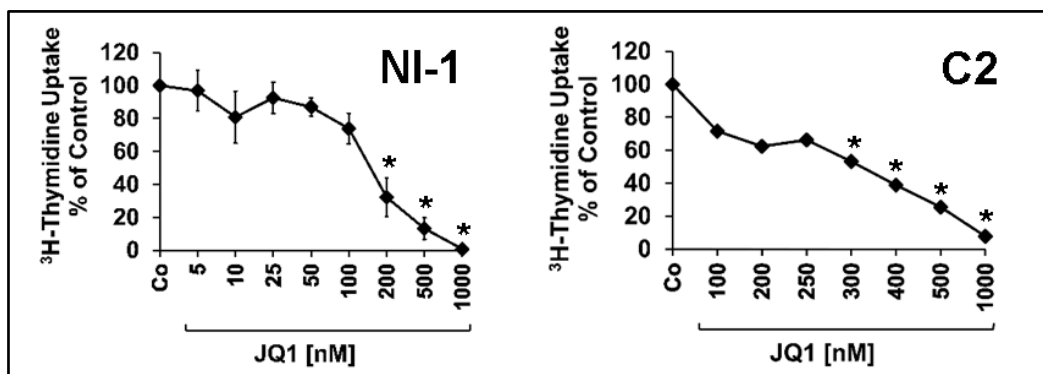


Figure S3: Effect of JQ1 on the proliferation of neoplastic canine MC lines. The canine MCL cell line NI-1 (left panel) and the canine mastocytoma cell line C2 (right panel) were incubated in the absence (Co) or the presence of various concentrations of JQ1 at 37°C for 48 hours. Then, ³H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to control (Co).

UNPUBLISHED SUPPLEMENTAL RESULTS

During our work, we have also generated several additional ROSA subclones:

- the ROSA^{KIT Δ 417-419 insY} cell line which presents with a very immature phenotype. This particular *KIT* defect is frequently found in pediatric patients suffering from cutaneous mastocytosis (Bodemer *et al.* 2010). Interestingly, we found that ROSA^{KIT Δ 417-419 insY} cells were negative for Fc ϵ RI and do not contain any cytoplasmic granulations upon MGG staining. The ROSA^{KIT Δ 417-419 insY} cells are SCF-independent like ROSA^{KIT D816V} cells, but were found sensitive to imatinib in contrast to ROSA^{KIT D816V} cells.
- the ROSA^{KIT K509I} cell line which displays a mature phenotype with higher surface expression of CD203c compared to ROSA^{KIT WT} cells. However, ROSA^{KIT K509I} cells are SCF-independent.
- More recently, we were also able to transduce ROSA^{KIT WT} cells with the following lentiviral constructs (gene-IRES-GFP) pWPI empty, pWPI HRAS WT and pWPI HRAS G12V to obtain three cell lines; ROSA^{pWPI empty}, ROSA^{HRAS WT} and ROSA^{HRAS G12V}. Only ROSA^{HRAS G12V} cells became SCF-independent and we observed that RAS was overexpressed in these cells (Figure S1).

Besides, regarding the involvement of BRD4 in the biology of neoplastic MCs, we were able to generate several additional data that have not been published yet.

For instance, we observed that BRD4 is expressed in the canine neoplastic MC lines NI-1 cells and C2 cells (Figure S2). Also, we found that BRD4-targeting drug JQ1 is able to inhibit ³H-thymidine uptake in NI-1 cells and C2 cells with IC₅₀ values of about 100-400 nM (Figure S3).

Besides, using RT-PCR, we observed that only the α chain of the Fc ϵ RI was significantly down-regulated in ROSA^{KIT WT} cells upon treatment with JQ1 (Figure S4). Moreover, we demonstrated that the treatment of ROSA^{KIT WT} with the proteasome inhibitors (MG-132 or bortezomib) was not able to compensate the JQ1-mediated down regulation of FcRI expression (Figure S5). Of note, the proteasome inhibitors (MG-132 or bortezomib) were found to exert inhibitory effects on the proliferation of both ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells with IC₅₀ <5 nM after 48 hours of

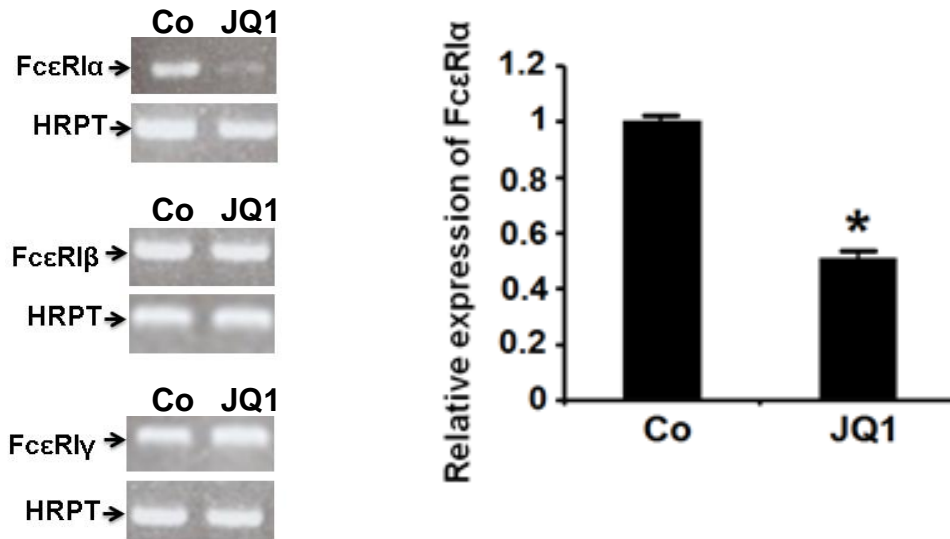


Figure S4: Effects of JQ1 on the expression of FcεRI subunits in ROSA cells. ROSA^{KIT WT} cells were incubated in control medium (Co) or in medium containing 200 nM of JQ1 at 37°C for 16 hours. Then, total RNA was extracted with trizol and cDNA was synthesized by RT-PCR as described (Wedeh *et al.* 2015). All PCR products were resolved by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. For quantitation, gels were scanned (left panels), and the relative level of FcεRIα band was quantified using the ImageJ software and normalized to the amount of HRPT (right panel). Results are shown as the mean±S.D. of the quantification of densitometry of three independent experiments. Asterisk (*) indicates $p < 0.05$ compared to untreated cells.

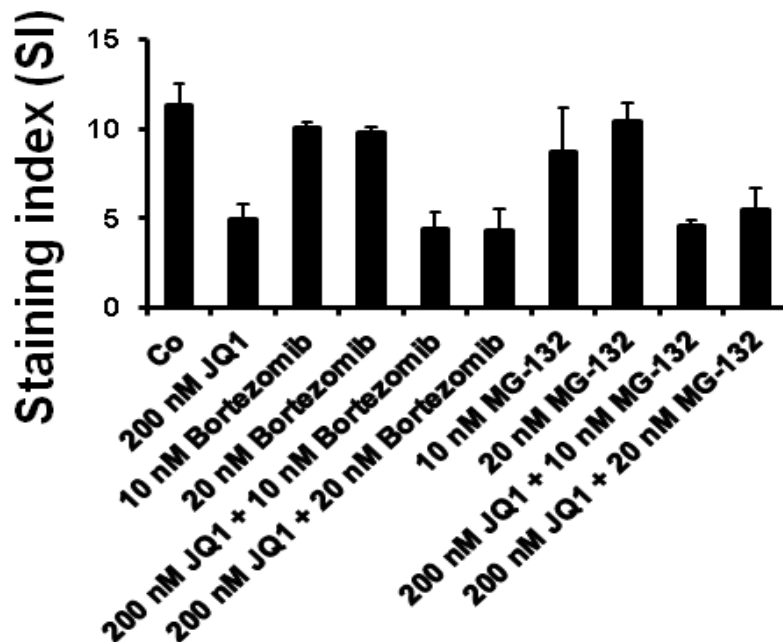


Figure S5: Effects of proteasome inhibitors on the expression of FcεRI in ROSA^{KIT WT} cells. ROSA^{KIT WT} cells were treated with 10 nM or, 20 nM of proteasome inhibitors (Bortezomib or MG-132) for 1 hour then incubated in medium or in medium containing 200 nM of JQ1 for 16 hours. Then, the expression of FcεRIα was analyzed by flow cytometry. Results are expressed as staining index (SI) and represent the mean±S.D. of three independent experiments.

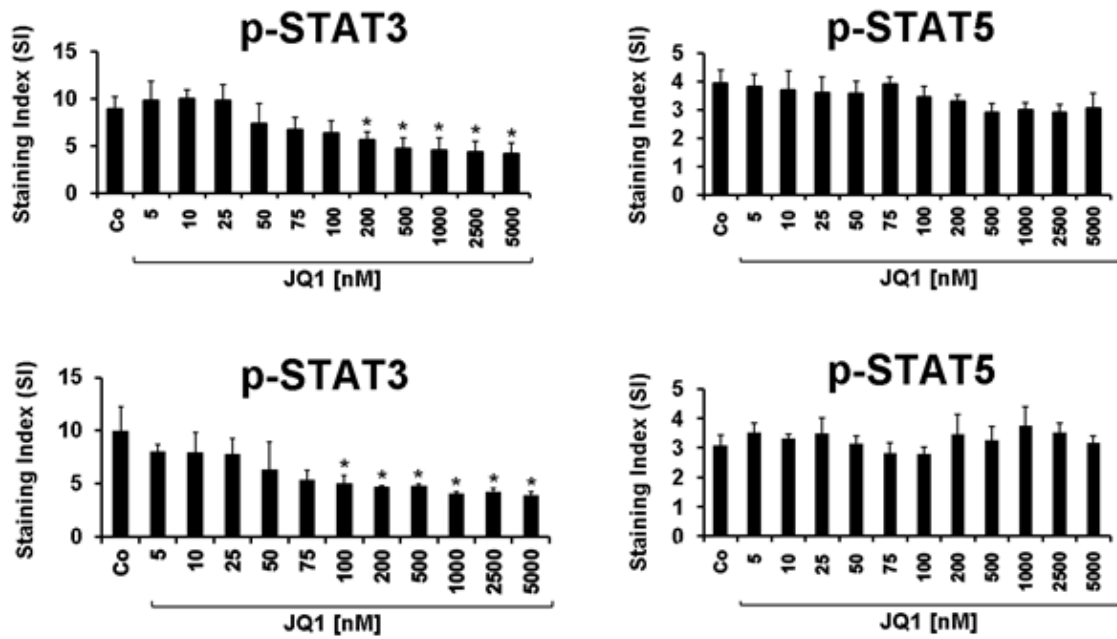
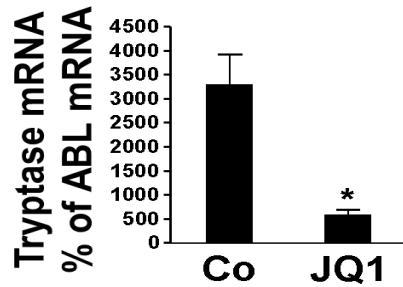


Figure S6: JQ1 suppresses p-STAT3 in ROSA^{KIT WT} cells. ROSA^{KIT WT} cells were incubated in medium (Co) or in medium containing different concentrations of JQ1 for 16 hours (upper panels) and 48 hours (lower panels). Thereafter, the intracellular expression of p-STAT3 (left panels) and p-STAT5 (right panels) was analyzed by flow cytometry as described in the section ‘Materials and Methods’. Results are expressed as SI and represent the mean±S.D. of three different and independent experiments. Asterisk (*) indicates p<0.05.

A



B

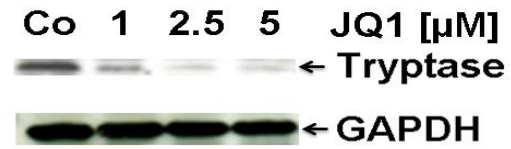


Figure S7: Effect of JQ1 on the expression of tryptase in human MC lines. A. $ROSA^{KIT WT}$ cells were incubated in control medium (Co) or medium containing JQ1 (2500 nM) at 37°C for 24 hours. Then, RNA was isolated and tryptase mRNA level was quantified by qPCR (left panel) as described (Wedeh *et al.* 2015). Results show transcript expression levels as percent of ABL mRNA expression and represent the mean \pm S.D of three independent experiments. Asterisk (*) indicates $p < 0.05$ compared to control. B. $ROSA^{KIT WT}$ cells were incubated in medium (Co) or in medium containing indicated concentrations of JQ1 for 24 hours (right panels) and then western blotting analysis for tryptase was conducted as described. GAPDH was probed to control for protein loading.

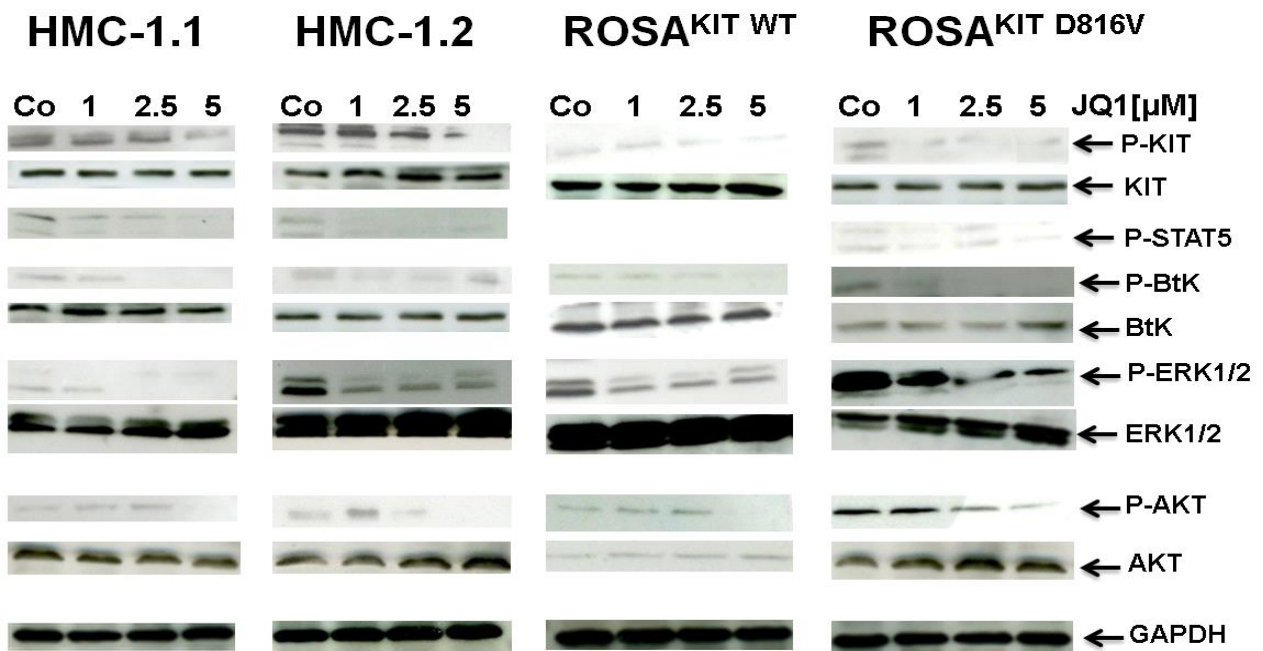


Figure S8: Effect of JQ1 on the expression of p-KIT, p-AKT, p-Btk, and p-ERK1/2 in human MC lines. HMC-1.1 cells, HMC-1.2 cells, $ROSA^{KIT WT}$ cells and $ROSA^{KIT D816V}$ cells were incubated in control medium (Co) or medium containing increasing concentrations of JQ1 (1000, 2500 and 5000 nM) at 37°C for 24 hours. Then, cells were lysed and western blotting analyses were performed using indicated antibodies as described above. Results show a representative blot and GAPDH was used as a loading control.

incubation. These data are in line with previously published reports which demonstrated that treatment of HMC-1.1 cells, HMC-1.2 cells and of the human leukemia cell line KU812 (established from the peripheral blood of a patient in blast crisis of chronic myelogenous leukemia) with different concentrations of hypomethylating agents (5-azacytidine and decitabine) had no effect on the surface expression of FcεRIα on these cells (Aichberger *et al.* 2009; Westerberg *et al.* 2012).

Additionally, we found that JQ1 was able to significantly decrease the expression of p-STAT-3 in ROSA^{KIT WT} cells in a dose dependent manner after 16 hours and 48, as shown in Figure S6, whereas the level of p-STAT-5 remained unaffected (Figure S6). Also, JQ1 was found to decrease the expression of tryptase in ROSA^{KIT WT} cells at both protein and mRNA levels (Figure S7).

Finally, as assessed by Western Blotting, we found that JQ1 decreased the phosphorylation of KIT in HMC-1.1 cells and HMC-1.2 cells as well as in ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells (Figure S7). In addition, JQ1 was found to attenuate p-AKT, p-BTK and p-ERK1/2 in a dose-dependent manner with no substantial effects on the expression of KIT, AKT, BtK and ERK1/2 in all human MC lines, as shown in Figure S8.

DISCUSSION

Despite the growing scientific advances, research on MCs suffered for long time from a lack of FcεRI+ human MC lines mimicking at the best the properties of normal MCs. In order to overcome this difficulty, we have recently isolated and characterized a new human MC line, derived from normal hematopoietic progenitors from a cord blood sample, that we termed ROSA^{KIT WT}. The ROSA^{KIT WT} cells are tryptase+ chymase- FcεRI+ SCF-dependent human MCs, with a KIT receptor which we showed to present with a normal sequence (Saleh, Wedeh *et al.* 2014). In addition, our cell line has a short doubling time, around 48-72h and is fully activable by a number of stimulants, including the cross-linking of their FcεRI. So far, only two FcεRI+ human MC lines have been established and published: LAD2 and LUVA cells (Kirshenbaum *et al.* 2003; Laidlaw *et al.* 2011). However, these two cell lines have several limitations, such as the long doubling time of about two weeks for LAD2 and the weak or rather negative surface expression of FcεRIα in LUVA cells. As described above, our ROSA^{KIT WT} cells have a short doubling time and stably express a functional FcεRI. Thus, these cells provide a novel advanced tool for MC research and enable the investigation of human MC functions as well as the high-throughput screening of anti-allergic therapeutics. Notably, the morphology and phenotype of these cells closely resemble the ones of primary cultured CBMCs, which is not the case for LUVA cells (Laidlaw *et al.* 2011). Interestingly, in contrast to cord blood-derived MCs (CBMCs), ROSA^{KIT WT} cells are easy to freeze and to thaw by conventional techniques and can be cultured for months without substantial changes in phenotype or significant alteration in their functional properties or clonal stability.

Since our laboratory is interested to work on the pathophysiology and treatment options of systemic mastocytosis, a category of disease characterized by multi-organ accumulation of neoplastic MCs harboring recurrently an activating point mutation in *KIT* (*KIT* D816V) (Valent 2008), we established the ROSA^{KIT D816V} cell line, a *KIT* D816V+ SCF-independent MC line which constitutes the first *in vitro* model of human MCs with a *KIT* D816V mutant alone and for which a *KIT* WT equivalent exists. Indeed, *in vitro* models yet available to study the impact of *KIT* D816V mutation on human MCs proliferation, survival and activation were limited. Of note, the only cell model available before

was the HMC-1 cell line and its two subclones HMC-1.1 cells and HMC-1.2 cells (Butterfield *et al.* 1988; Sundström *et al.* 2003). Both clones contain the *KIT* V560G mutation, whereas HMC-1.2 additionally exhibits the *KIT* D816V mutation. Unfortunately, HMC-1 cells do not bear the *KIT* D816V alone and there is no HMC-1 subclone with a normal *KIT* gene, making it difficult to compare signaling recruited by normal or mutant forms of *KIT* in the same cell model.

Interestingly, we observed that our ROSA^{KIT D816V} cell line, which expresses CD117, CD203c, and tryptase although not expressing CD2 and CD25, acquired *in vitro* (and *in vivo*, see below) the morphological phenotype of neoplastic MCs found in patients suffering of SM, i.e.; a spindle-shaped appearance (Valent *et al.* 2007), which is not the case for any other *in vitro* models of human mastocytosis.

Besides, activation of KIT recruits a number of signaling pathways in MCs. Specifically, activation of the PI3-K/AKT pathway was shown to contribute to KIT-dependent proliferation, survival, maturation, adhesion, or activation (Ma *et al.* 2011). Interestingly, the introduction of *KIT* D816V in ROSA^{KIT WT} cells induced the constitutive activation of AKT, which is in line with previously reported data (Casteran *et al.* 2003). In addition, KIT D816V recruits alternative signaling pathways not evoked by KIT WT, particularly STAT5 (Mayerhofer *et al.* 2008; Baumgartner *et al.* 2009; Chaix *et al.* 2011). Interestingly, we observed that ROSA^{KIT D816V} cells constitutively expressed p-STAT5, suggesting that they use the same alternative pathways as MCs in mastocytosis patients.

Surprisingly, we observed that, although expressing FcεRI at the same levels than ROSA^{KIT WT} cells, ROSA^{KIT D816V} cells do not respond to anti-IgE-mediated activation as neither do the BM-derived neoplastic MCs from patients with ASM and MCL (Saleh, Wedeh *et al.* 2014). The mechanism(s) involved in such hyporesponsiveness is still unknown. However, Ito *et al.* have recently shown that MCs chronically exposed to SCF display a marked attenuation of FcεRI-mediated degranulation and cytokine production (Ito *et al.* 2012). Such desensitization may also play a role in *KIT* D816V-positive cells in which the KIT receptor is chronically phosphorylated. Since FcεRI and KIT use common signaling pathways, cross-desensitization may occur (Hundley *et al.* 2004). Alternatively, KIT D816V may induce the expression of negative regulators of MC activation, such as phosphatases. All in all,

these findings might explain why many patients with SM, even with high MC burden, do not suffer at all from IgE-related mediator-related symptoms. Nevertheless, a number of patients may suffer from mediator-release symptoms related to non-IgE triggers, such as drugs, infection, or alcohol (Brockow & Metcalfe 2010).

In addition, we confirmed that ROSA^{KIT D816V} cells were unresponsive to antiproliferative effects of imatinib, a tyrosine kinase inhibitor which targets BCR/ABL1 and wild-type KIT, but previously shown devoid of significant effects on the TK activity of the KIT D816V mutant protein (Ustun *et al.* 2011). However, as expected, the ROSA^{KIT D816V} cells remained sensitive to dasatinib and to midostaurin, two TKIs with broad activity of a variety of TKs, including KIT D816V mutant receptor (Saleh, Wedeh *et al.* 2014). Thus, given its ability to be grown easily in large amounts, the ROSA^{KIT D816V} cell line can be used to perform a high-throughput screening of TKIs selectively targeting the KIT D816V mutations drugs *in vitro* as well as *in vivo* after injecting these cells to mouse models.

As of today, there has been several attempts to establish *in vivo* models mimicking aggressive SM using various techniques and protocols, with more or less success. For instance, the HMC1.2 cell line (Butterfield *et al.* 1988) has been injected in nude mice, and this gave rise to solid mast cell tumors (Schumacher *et al.* 1998). However, HMC1.2 is a human mast cell line derived from a patient with a MCL, the cells contain 80 chromosomes and present with two *KIT* mutations (which is unusual in SM patients). Thus this model looks far from ASM in humans for all these reasons. Besides, another model was made by injecting P815 cells injected in DBA2 mice (Demehri *et al.* 2006). P815 is a mouse mastocytoma cell line which harbors a D814Y mutation in a mouse *Kit* gene (corresponding to a human *KIT* D816Y and not D816V). When injected IV in DBA2 mice, it gives rise mostly to MCL. In this case the mice died in 7-8 days. Clearly all these elements make the model far from ASM patients.

Later, our team was involved in the development of a mouse model transgenic for the human *KIT* D816V gene, which was selectively expressed in MCs because the gene coding for the *KIT* mutant was expressed under a baboon chymase promoter (Zappulla *et al.* 2005). This seemed to us a good model to study the pathophysiology of mastocytosis, although we observed that a SM-like disease developed only in one to 2 years and in only one third of the transgenic animals (Zappulla *et al.* 2005).

In addition, while in this model the *KIT* mutant was of human nature, it was expressed in murine mast cells. Finally, this model appears clearly not suitable for *in vivo* assay of drugs given the time needed to observe appearance of the disease and the frequency of diseased animals (Zappulla *et al.* 2005).

A more recently published *in vivo* model of mastocytosis consisted of mice engineered to express conditionally a constitutively active *Kit* D814V murine mutant, i.e; a Cre/loxP-based bacterial artificial chromosome transgenic mouse model (Gerbaulet *et al.* 2011). Although the model is still dealing with mouse mast cells harboring a murine *Kit* mutant, which is far from the reality of neoplastic MCs cells in ASM patients (a human *KIT* D816V mutant in human MCs), however, this model has some merits since, in certain conditions, by crossing *KitD814Vflox* mice to *A-Mcpt5-Cre* line (Gerbaulet *et al.* 2011), which deletes loxP-flanked DNA, the *Kit* D814V mutant can be driven to be selectively expressed only in mature MCs. This gives rise to a slowly progressive SM, with bone marrow involvement and with a latency of up to 52 weeks. In addition, affected mice suffered of severe colitis. Although potentially interesting to analyze the pathophysiology of mastocytosis, this model seems not fully suitable for drug testing for several reasons such as the latency in the development of the disease (one year, which is not fully compatible with drug testing).

Given that the *in vivo* models of mastocytosis yet available were not fully comparable to what is observed in the human SM patients, for the reasons exposed above, we sought to investigate whether we could create, using our $\text{ROSA}^{\text{KIT D816V}}$ cell line, a new and more relevant *in vivo* model of mastocytosis mimicking at the best the human disease, in order to be able not only to investigate the pathophysiological events involved in the development of the disease, but also to screen for drugs potentially active *in vivo* against the KIT mutant or the signalling pathways evoked by the aberrantly activated TK. Interestingly, we were able to show that, after intravenous injection in the tail vein of NOD/SCID IL-2R $\gamma^{-/-}$ (NSG) mice, $\text{ROSA}^{\text{KIT D816V}}$ cells can engraft at a high rate in these animals, not only in the BM, but also in spleen and lung, giving rise to a disease mimicking at best the situation encountered in SM patients (Saleh, Wedeh *et al.* 2014). Indeed, as evidenced by immunohistochemistry for human tryptase on BM sections of NSG mice injected intravenously with $\text{ROSA}^{\text{KIT D816V}}$ cells, compact infiltrates of tryptase-positive human MCs can be seen (Saleh, Wedeh *et*

al. 2014). Together with the spindle-shaped appearance of the neoplastic MCs found on BM smears of the animals, and with their positivity for *KIT* D816V, our animal model exhibits several features resembling SM in humans, making it the most similar model for human SM described so far. Both ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells have been patented (international approved patent WO/2013/064639) and published (Saleh, Wedeh *et al.* 2014).

The *KIT* D816V mutation is known to confer resistance against imatinib (Gotlib *et al.* 2006; Ustun *et al.* 2011). So far, no curative treatment is available for patients with advanced SM (Arock *et al.* 2015). In these patients, no standard treatment exists and cytoreductive therapy is usually required to keep the disease process under control (Valent *et al.* 2010; Arock *et al.* 2015). Besides antimediation therapy, and depending on the aggressiveness of the disease, targeted or non-targeted chemotherapies, such as IFN- α , 2-CdA, or hydroxyurea (HU), or even allogeneic stem cell transplantation (allo-SCT) are sometimes required to control the end-organ damage caused by MC infiltration (Siebenhaar *et al.* 2014; Arock *et al.* 2015). Despite the encouraging data obtained in clinical trials with the TKI midostaurin (PKC412) in patients with advanced SM, neoplastic MCs may bypass and rely on KIT-independent pathways for survival (Gleixner *et al.* 2011; Arock *et al.* 2015). Therefore, considerable efforts and investigations are made to develop drugs targeting the oncogenic signaling machinery downstream of KIT and/or other, KIT-independent survival pathways

Moreover, given that there is also an increasing need to identify other druggable targets in neoplastic MCs from patients with advanced SM, we were interested to evaluate the potential activity of BET inhibitors in neoplastic MCs *in vitro* and *ex vivo*. In fact, bromodomain-containing proteins have recently been reported to be of interest in several hematological malignancies and in solid tumors (Filippakopoulos *et al.* 2010; Zuber *et al.* 2011; Delmore *et al.* 2011; Mertz *et al.* 2011; Dawson *et al.* 2011; Lockwood *et al.* 2012; Herrmann *et al.* 2012; Puissant *et al.* 2013; Bandopadhyay *et al.* 2014; Shi *et al.* 2014; Asangani *et al.* 2014). These proteins have also been recently identified as key transcriptional regulators of cell cycle progression and growth in various neoplastic cells (Zuber *et al.* 2011; Herrmann *et al.* 2012; Dawson *et al.* 2012; Godley & Le Beau 2012; Belkina & Denis 2012). One of these regulators appears to be BRD4, an epigenetic reader involved in the regulation of MYC.

Moreover, it has been recently established that BRD4 is a druggable epigenetic target in AML cells (Zuber *et al.* 2011; Herrmann *et al.* 2012) and similar observations have been made in other tumor models (Ott *et al.* 2012; Fiskus *et al.* 2014; Roderick *et al.* 2014). BRD4 inhibition by the selective BET bromodomain inhibitor and the BRD4-targeting drug JQ1 has been reported to abrogate tumor growth *in vitro* as well as *in vivo* (Filippakopoulos *et al.* 2010). In addition, it has been recently shown that BRD4 is expressed abundantly in the cytoplasm of leukemic blast cells in AML (Herrmann *et al.* 2012). Consistently, in the present study, we have showed that BRD4 protein is highly expressed in the cytoplasm of neoplastic MCs derived from patients with advanced SM, and that the BRD4-targeting drug JQ1 induced growth inhibition and apoptosis in these cells (Wedeh *et al.* 2015). More precisely, we were able to show that BRD4 is expressed abundantly in the cytoplasm of neoplastic MCs in almost all patients with advanced SM, whereas in most patients with ISM, MCs expressed only low levels or no detectable the BRD4 protein in their cytoplasm. In addition, we were also able to demonstrate that HMC-1 cells as well as ROSA cells expressed cytoplasmic and nuclear BRD4 (Wedeh *et al.* 2015).

Of interest, BRD4 knock-down by RNA silencing in human neoplastic MC lines decreased the proliferation of these cells, and induced apoptosis. In addition, we observed that RNA silencing of BRD4 was associated with the downregulation of *MYC* mRNA. These data strongly suggest that BRD4 protein likely harbors functions that are crucial for the survival of neoplastic MCs. As it is well known that ASM and MCL are incurable malignancies characterized by drug resistance and a poor survival (Travis *et al.* 1986; Valent *et al.* 2001; Horny & Valent 2001; Sperr *et al.* 2001; Valent *et al.* 2003; Akin & Metcalfe 2004; Valent *et al.* 2004; Valent *et al.* 2004; Horny *et al.* 2007; Metcalfe 2008; Lim *et al.* 2009; Arock & Valent 2010), it was critical to learn more about new drug targets expressed in neoplastic MCs and to identify more effective targeted drugs that have the potential to overcome drug resistance in ASM and MCL. In the present study, we have identified BRD4 as a novel drug target in neoplastic MCs in advanced SM (Wedeh *et al.* 2015). To the best of our knowledge this is the first report describing a BET bromodomain-containing molecule as a regulator of cell growth and therapeutic target in neoplastic MCs.

The expression of cytoplasmic BRD4 protein in neoplastic MCs in advanced SM may have several explanations. One hypothesis is that cytoplasmic expression of BRD4 is indicative of a higher proliferative potential of neoplastic MCs. An alternative explanation would be that additional, *KIT*-independent, lesions acquired during disease progression from indolent to advanced SM, promote cytoplasmic expression of BRD4 in neoplastic MCs. Indeed, apart from *KIT* D816V, several additional molecular lesions and pro-oncogenic signaling pathways are expressed and activated in neoplastic MCs in advanced SM.

Although the exact role and biochemical function of cytoplasmic BRD4 in neoplastic MCs remain at present unknown, it is interesting to notice that BRD4 is considered to display a kinase activity (Devaiah *et al.* 2012) and to interact with a number of cytoplasmic antigens, such as the signal-induced proliferation-associated protein 1 (SPA-1) in malignant cells (Gleixner *et al.* 2011; Wilson *et al.* 2011; Schwaab *et al.* 2013). BRD4 interacts with SPA-1 *in vivo* and *in vitro* and increases Rap GAP activity of SPA-1. In addition, BRD4 and the SPA-1 molecules have been documented to regulate each other's subcellular localization and to affect cell cycle progression from G₂ through M (Farina *et al.* 2004). Whether BRD4 also fulfils these functions in the cytoplasm of neoplastic MCs remains unknown. Thus, further studies are required to understand in more details the precise role of BRD4 in the context of its cytoplasmic expression in neoplastic MCs of advanced SM.

Regarding the mechanisms contributing to the expression of BRD4 in neoplastic MCs, little was known so far. In order to investigate the signaling pathways potentially involved in the expression of BRD4, we performed experiments using various signal transduction inhibitors, including TKIs such as imatinib, dasatinib, PKC412, and ponatinib, MEK inhibitors, PI3K/mTOR inhibitor, ATRA, 2CdA, demethylating agents, JAK2 inhibitors, and STAT inhibitors piceatannol and pimozone. In these experiments, the multi-targeted TKIs PKC412 and dasatinib, that are known to block the kinase activity of *KIT* D816V, were found to downregulate the expression of BRD4 in HMC-1.1 cells, HMC-1.2 cells, ROSA^{KIT WT} cells and ROSA^{KIT D816V} cells. In addition, we found that various MEK inhibitors and the PI3K/mTOR blocker BEZ235 can downregulate expression of BRD4 in neoplastic MCs. Since MEK as well as PI3-K are downstream effectors of *KIT* (Hu *et al.* 2003; Roskoski 2005; Harir *et al.*

2008), these data suggest that KIT D816V but also other KIT mutants and even KIT WT may contribute to the expression of BRD4 protein in neoplastic MCs. This hypothesis is supported by the observation that SCF promoted the expression of BRD4 in ROSA^{KIT D816V} cells. However, SCF failed to upregulate expression of BRD4 in HMC-1 cells. These observations suggest that expression of BRD4 in MCs depends not only on KIT activation, but also on other factors. Moreover, it has been recently shown that the BRD4-targeting drug JQ1 exerts strong anti-neoplastic effects in drug-resistant AML cells (Sotlar *et al.* 2003; Herrmann *et al.* 2012). In the present study, we were able to show that JQ1 induces growth inhibition and apoptosis in HMC-1 cells and ROSA cells as well as in primary neoplastic mast cells obtained from patients with advanced SM. Interestingly, in each cell line tested, the effects of JQ1 on malignant cell growth were dose-dependent, with reasonable IC₅₀ values, and in each case, growth-inhibition was associated with induction of apoptosis. In addition, the BRD4-targeting drug JQ1 was found to block in a dose-dependent manner the cell cycle progression in all human MC lines tested in our study. Moreover, we found that BRD4 is overexpressed in the cytoplasm of the canine neoplastic mast cell lines NI-1, harboring several homozygous *Kit* mutations, including missense mutations at nucleotides 107(C⇒T) and 1187(A⇒G), a 12-bp duplication (nucleotide 1263), and a 12-bp deletion (nucleotide 1550) and the C2 mastocytoma cell line harboring a *Kit* exon 11 mutation (DeVinney & Gold 1990; Hadzijušufovic *et al.* 2012), and that inhibition of BRD4 using JQ1 was found to block the proliferation of these canine cell lines in a dose-dependent manner. As well, JQ1 induced a dose-dependent apoptosis in the same cell lines. To the best of our knowledge this is also the first demonstration that BRD4 inhibition may impede dog neoplastic MC proliferation and survival. This might have interesting therapeutic consequences at the level of veterinary medicine since, by contrast to the human situation, mastocytoma is one of the most common malignant disease found in dogs and has always a poor prognosis in such animals (Cohen *et al.* 1974; London & Sequin 2003; Misdorp 2004; Welle *et al.* 2008; Hosseini *et al.* 2014).

In AML, JQ1 effects were found to be associated with signs of maturation (Zuber *et al.* 2011; Herrmann *et al.* 2012). We were therefore interested to explore whether exposure to JQ1 could lead to maturation in HMC-1 cells and ROSA cells. However, we were not able to detect a morphologically

visible maturation-inducing effect of JQ1 on these cells. Moreover, we were unable to demonstrate that JQ1 induced upregulation of differentiation-associated antigens in the same cells. Rather, we observed that JQ1 downregulated the expression of several key surface antigens in HMC-1 cells and/or ROSA cells, including CD63 (LAMP-3) and CD71 (transferrin receptor). Besides, JQ1 was found to downregulate the expression of FcεRI on ROSA, which may point to the fact that FcεRI expression is regulated by epigenetic mechanisms in these cells. To the best of our knowledge, this is the first report describing that FcεRI expression in human MCs is regulated by an epigenetic mechanism involving a bromodomain-containing molecule. We were also able to show that this effect of JQ1 is of functional significance. Indeed, JQ1 was found to suppress FcεRI-dependent histamine release in ROSA^{KIT WT} cells as well as IgE-anti-IgE-induced upregulation of CD63 and CD203c in these cells, presumably because of down-regulation of FcεRI expression. Also, interestingly, we found that expression of tryptase decreased in ROSA^{KIT WT} cells upon treatment with JQ1.

Besides, JQ1 was found not only to abrogate the expression of p-STAT3 in ROSA^{KIT WT} cells but also to decrease the expression of p-STAT5 in ROSA^{KIT D816V} cells, HMC-1.1 cells and HMC-1.2 cells in a dose-dependent manner. These observations are consistent with a recent report in which JQ1 was shown to inhibit p-STAT5 in myeloid malignancies (Liu *et al.* 2014). Collectively, these data suggest that JQ1 blocks several molecular key programs involved not only in the proliferation, but also in the activation of neoplastic MCs, which may be relevant clinically, as patients with advanced SM not only suffer from organ infiltration but also from the consequences of MC activation and mediator release (Valent *et al.* 2001; Valent *et al.* 2004; Horny *et al.* 2007; Metcalfe 2008; Arock & Valent 2010). We then examined if the treatment of ROSA^{KIT WT} cells with proteasome inhibitors might rescue JQ1-induced downregulation of FcεRIα surface expression on these cells. However, no effect of proteasome inhibitors was found in this context. Instead, we found that these proteasome inhibitors exert antiproliferative effects on these cells, with an IC50 of about 5 nM. In addition, these antiproliferative effects were associated with induction of apoptosis not only in ROSA^{KIT WT} cells but also in ROSA^{KIT D816V} cells.

Interestingly, using RT-PCR experiments, we were able to show that JQ1 induced the downregulation

of the expression of mRNA coding for FcεRIα in ROSA^{KIT^{WT}} cells. In addition, using qPCR, we found that JQ1 treatment decreased the level of transcription of MYC as well as of BCL-2, BCL-xL and MCL-1, whereas it increased the transcription of the proapoptotic molecule BIM in human MC lines. These observations are consistent with the recently published reports describing that JQ1 can inhibit the binding of BRD4 and of pTEFb to the super enhancers of some oncogenes including MYC, and BCL-2, leading to the depletion of their transcript levels (Delmore *et al.* 2011; Lee & Young 2013; Lovén *et al.* 2013; Chapuy *et al.* 2013). Figure 25 shows a scheme recapitulating the effects of JQ1 in neoplastic MCs.

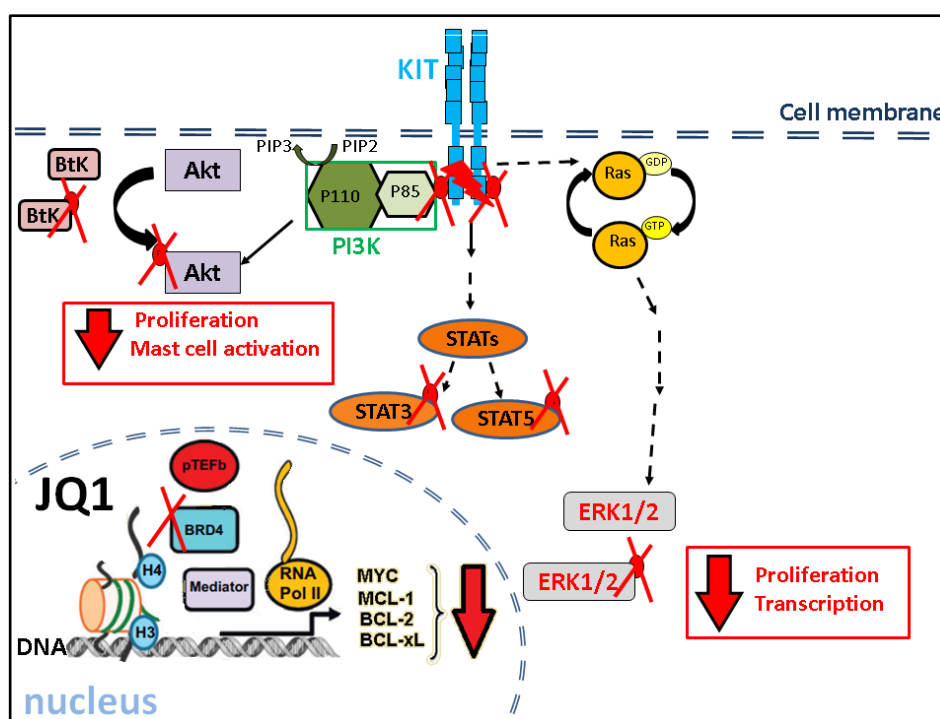


Figure 25: Molecular scheme demonstrating the effects of JQ1 on neoplastic MCs. The figure shows the major signaling pathways evoked by the KIT D816V mutant receptor in neoplastic MCs. In this scheme, BRD4 binds to acetylated histones and recruits pTEFb to the enhancer/promoter of target genes such as c-MYC, and BCL-2. Upon its recruitment by BRD4, pTEFb phosphorylates the CTD of RNA POL II, resulting in transcription of target genes. Treatment with BET protein inhibitors JQ1 inhibits BRD4 activity causing depletion of RNA POL II phosphorylation at Ser 2, reduced binding of BRD4 and RNA POL II at target gene promoters, and decreased transcription of target genes (MYC, BCL-2, BCL-xL, and MCL-1). In addition, treatment with JQ1 inhibits cell growth and induces apoptosis of neoplastic MCs. Treatment with JQ1 also decreases FcεRI expression, IgE-mediated histamine release, CD63, CD71, and the activation of KIT, STAT3, STAT5, AKT, BTK and ERK1/2.

In addition, since demethylating agents have been recently shown to lead to over-expression of FcεRI on monocytes of patients with atopic dermatitis (Liang *et al.* 2012), we were thus interested to know whether treatment of HMC-1.1 cells, HMC-1.2 cells and KU812 cells (which usually lack the

expression of this receptor) with these agents would induce expression on FcεRI at the cell surface. However, we found that demethylating agents do not induce FcεRI surface expression in these cells. Moreover, the analysis of the data obtained after immunoblot conducted on HMC-1.1, HMC-1.2, ROSA^{KIT WT}, and ROSA^{KIT D816V} cells upon treatment with JQ1 led us to conclude that the MEK and PI3-Kinase pathways are involved in the mechanism underlying the antineoplastic effect of the drug on these cells. Therefore, our hypothesis is that the RAS signalling pathway might be involved in this effect. Our findings are consistent with several recent reports in the context of the activity of BRD4 inhibitors in KRAS-positive tumours in other models (Shimamura *et al.* 2013; De Raedt *et al.* 2014; Roy *et al.* 2015. Borbely *et al.* 2015). For instance, changes in the epigenetic state in a KRAS-driven pancreatic cancer formation model initiate duct-derived intraductal papillary mucinous neoplasia (IPMN) lesions (Roy *et al.* 2015). Also, JQ1 treatment impairs the tumorigenicity of *Kras* G12D, pancreatic ductal adenocarcinoma (PDA) and (IPMN)-derived murine cancer cell lines both *in vitro* and *in vivo* (Roy *et al.* 2015). Moreover, JQ1 abrogates the survival and the viability of KRAS-positive non-small cell lung cancer (NSCLC) cell lines. However, the activity of JQ1 in mutant KRAS NSCLC is abrogated by concurrent alteration or genetic knock-down of LKB1 (Shimamura *et al.* 2013). Similarly, JQ1 treatment produces significant tumor regression in mutant *kras* mice, whereas, tumors from mutant *kras* and *lkb1* mice did not respond to JQ1 (Shimamura *et al.* 2013). Interestingly, by amplifying Ras-driven transcription through effects on chromatin, the loss of the tumour suppressor polycomb group gene *SUZ12* potentiates the effects of mutations in *NFI*, which encodes a Ras GTPase-activating protein (RasGAP) and whose loss drives cancer by activating Ras (De Raedt *et al.* 2014). Importantly, *SUZ12* inactivation also triggers an epigenetic switch that sensitizes peripheral nerve sheath tumours to bromodomain inhibitors (De Raedt *et al.* 2014). More recently, co-treatment with JQ1 and the HDAC inhibitor was showed to reduce breast cancer cell viability through induction of the expression of several members of the ubiquitin-specific protease 17 (USP17) family of deubiquitinating enzymes that attenuate the Ras/MAPK pathway causing decrease in cell viability (Borbely *et al.* 2015).

Based on these studies, we have recently generated ROSA^{HRAS WT} and ROSA^{HRAS G12V} cell lines, which

will enable us to further investigate the validity of our hypothesis about the involvement of RAS in the activity of BRD4. ROSA^{HRAS WT} cells overexpress the RAS WT but remain SCF-dependent, whereas ROSA^{HRAS G12V} cells are SCF-independent. We will compare the proliferation rate and the expression of BRD4 in both cell lines, as well as the effects of JQ1 on cell proliferation and apoptosis. It is here tempting to speculate that overexpression of RAS or RAS mutation will confer sensitivity to JQ1 in ROSA SCF-mediated resistant cells.

Whatsoever, the intriguing effects of JQ1 on growth and survival of neoplastic MCs prompted us to search for optimal drug partners through which synergistic anti-neoplastic effects can be obtained. To address this point, we tested a number of different combination partners in HMC-1 cells and ROSA cells. Of all drug-partners tested, PKC412 and ATRA were found to synergize with JQ1 in inhibiting proliferation and survival in HMC-1 cells and ROSA cells, which may have clinical implications. Of note, PKC412 is already in clinical trials for the treatment of patients with Advanced SM and seems to present beneficial effects in such cases (Gotlib *et al.* 2014), whereas it has been already shown that ATRA may negatively impact the development of normal and malignant MCS (Ishida *et al.* 2003; Hjertson *et al.* 2003; Alexandrakis *et al.* 2003; Ohashi *et al.* 2006; Pinello *et al.* 2009). Thus, that JQ1 synergizes with such drugs in inhibiting the growth of human malignant MC lines was expected.

In a final step, we asked whether resistance against JQ1 may occur in neoplastic MCs. In these experiments, we found that SCF introduced resistance against JQ1 in ROSA^{KIT D816V} cells but not in HMC-1 cells. Although the underlying mechanism of this drug resistance induced by the addition of SCF in ROSA cells remains at present unknown, we asked whether the synergistic activity of the drug combinations JQ1+ATRA and JQ1+PKC412 would overcome this SCF-mediated drug resistance. Interestingly, we found that the combination JQ1+PKC412, but not of JQ1+ATRA, was able to overcome SCF-mediated resistance in ROSA cells. Given these data, one can hypothesize that the SCF-induced resistance to JQ1 is mediated by over-activation of the KIT receptor by its ligand, and that this mechanism of resistance can only be overcome by the addition of a strong KIT-blocking agent such as PKC412.

In summary, our data show that BRD4 is a promising druggable target in neoplastic MCs in advanced

SM. Our data also show that inhibition of BRD4 by JQ1 is a potent approach to eradicate neoplastic MCs, and that these interesting drug effects could be potentiated by addition of PKC412 or of ATRA.

CONCLUSIONS AND PERSPECTIVES

In the first part of our work, we have been able to establish a new human FcεRI+ Tryptase + MC line exhibiting the major characteristics of normal MCs, termed ROSA^{KIT WT} cells. Using this cell line, which is SCF-dependent, we were able to show its full activation capabilities after cross-linking of their FcεRI. This cell line, which has a short doubling time and a stable phenotype, represents a significant advance towards other human mast cell lines already available. In addition, using lentiviral infection of these ROSA^{KIT WT} cells, we have been capable to generate a new stable subclone, termed ROSA^{KIT D816V} cells, since the cells were transduced with a construct expressing the *KIT* D816V mutant. This new subclone shares a number of characteristics with neoplastic MCs found in aggressive SM (ASM), including growth-factor independence for its proliferation and constitutive activation of KIT and of downstream signaling pathways already known to be evoked in neoplastic MCs from SM patients, such as STAT-5 and AKT. Moreover, after intravenous injections, these ROSA^{KIT D816V} cells were able to produce a systemic mastocytosis-like disease in NSG mice, with an aggressive phenotype. ROSA^{KIT D816V} cells can thus be used for testing new anti-neoplastic drug therapies *in vitro* and *in vivo* and can be helpful to fully understand the pathogenicity of the disease. Finally, this malignant cell line can be easily manipulated and used to introduce the more frequent additional genetic alterations found in ASM, such as mutations in *TET2*, *SF3B1*, etc...with the aim to better understand how these somatic mutations alter the behavior and prognosis of the disease.

Given that patients with advanced categories of SM have a poor prognosis and that the therapeutic arsenal for such patients is still limited, we were thus interested, in the second part of our work, to find new targets of potential therapeutic interest in neoplastic MCs. Starting from the literature already available showing a potential role of BRD4 in several hematological malignancies, we decided to investigate the possible role of this molecule in SM. Interestingly, we have been able to show a significant expression of cytoplasmic BRD4 protein in human and canine neoplastic MC lines. In addition, we have demonstrated that neoplastic MCs derived from patients with ASM and MCL overexpress BRD4 in their cytoplasm. Moreover, we have showed that RNA silencing of BRD4 in human MC lines inhibited the proliferation of these cells, and induced their apoptosis with

downregulation of MYC transcript. However, further studies are still required to determine the specific role and the particular biochemical functions of BRD4 aberrantly localized in the cytoplasm of neoplastic MCs in SM. Moreover, the interaction of cytoplasmic BRD4 with other cytoplasmic antigens, such as SPA-1, in neoplastic MCs remains to be elucidated. These studies may reveal other promising novel and druggable targets in neoplastic MCs and their progenitors, leading to an optimization of the therapeutic options with the aim of increasing the survival time of patients with advanced SM.

Whatsoever, the relevance of our data obtained on neoplastic MC lines was confirmed by further experiments performed with the use of JQ1, a first-in-class prototype of bromodomain inhibitor (a benzodiazepine-based drug developed in Bradner's laboratory). Indeed, inhibition of BRD4 using this molecule, leads in our hands to sustained inhibition of proliferation associated with the induction of apoptosis in human neoplastic MC lines as well as in primary MCs derived from patients with ASM and MCL. In addition, JQ1 was found to block dose-dependently the cell cycle progression in human malignant MC lines and to decrease in the same way the surface expression of CD63 and CD71 on these cells.

One intriguing observation made during our experiments was the fact that JQ1 potently downregulated the expression of FcεRI in ROSA^{KIT} WT cells and suppresses FcεRI-dependent histamine release and IgE-anti-IgE-induced upregulation of CD63 and CD203c in these cells. This finding underlines the therapeutic potential of BRD4 inhibition in neoplastic MCs derived from patients with advanced SM suffering from the consequences of MC activation and mediator release. Indeed, it appears that JQ1 may not only inhibit the proliferation of neoplastic MCs, but might also decrease the exaggerated release of MC-derived mediators in SM patients who are prone to develop MC mediators symptoms. However, this interesting effect of JQ1 on FcεRI expression and on IgE-anti-IgE-dependent histamine release deserves further evaluation in human normal MCs and basophils. Particularly, the molecular mechanisms involved in such effects need to be further investigated and, among others, effects of JQ1 on the expression and phosphorylation state of LYN, SYK or PLC-γ need to be evaluated on resting and FcεRI-activated cells.

Interestingly, we observed that JQ1 treatment decreased transcriptional levels of MYC levels. This observation is consistent with published data on the effects of the same molecule in other cancer cells. Thus, one can hypothesize that JQ1 treatment could thereby suppress transcription of MYC-dependent target genes in neoplastic MC lines. However, this needs to be confirmed in further experiments.

Although the BET bromodomain is primarily known to associate with chromatin through protein-protein interaction with acetylated histones, our data support the fact that JQ1 inhibits the activation of STAT3 and STAT5 in human MC lines. In addition, we also demonstrated that JQ1 blocks PI3-Kinase and MEK pathways involved in the survival and proliferation of neoplastic MCs. These observations may be important to identify other druggable targets.

Moreover, we found that SCF introduced resistance against JQ1 in ROSA^{KIT D816V} cells. The underlying mechanism(s) of this SCF-induced resistance in these cells remain(s) at present unknown and require(s) further investigations. Whatsoever, we observed that SCF promoted the expression of BRD4 at the transcriptional level in these cells, underlining the potential involvement of this cytokine in the context of proliferation and survival of neoplastic MCs. However, cytokine-induced resistance to JQ1 might be due to mutations in BRD4 developed as a mechanism of resistance in neoplastic MCs, indicating the necessity to rule out these mutations. Genome-wide transcriptome analysis comparing cells treated with control or with JQ1 in the presence and absence of SCF could be of great interest in order to uncover alternate and possible mechanisms of resistance. Importantly, the synergistic activity of the JQ1 with PKC412 can overcome SCF-mediated JQ1 resistance in neoplastic MCs. Indeed, the combination JQ1+PKC412 was found to overcome SCF-mediated resistance in ROSA cells.

Alltogether, our data show that BRD4 is a promising druggable target in neoplastic MCs in advanced SM. BRD4 inhibition provides an opportunity to target dysregulated transcriptional programs in neoplastic MCs. Of note, JQ1 has been reported to show reasonable safety profile and tolerance in mice. However, the potency of BRD4 inhibition with JQ1 or other I-BET *in vivo* models such as NSG mice and in clinical trials in patients with advanced SM is still unknown. Hitherto, the antineoplastic effect observed with JQ1 or other BET-bromodomain inhibitors *in vivo* and in patients with advanced

SM remains also to be elucidated.

In conclusion, our data highlight the promise of BET protein antagonists for the treatment of patients with advanced SM. However, while current BET inhibitors target simultaneously BRD2, BRD3 and BRD4, it might be beneficial to design more specific and selective inhibitors for BRD4, as well as to develop chemical tools that could allow to discriminate between the disease-relevant functions of each bromodomain present (BD1 and BD2) in BRD4. Continued research efforts are also needed in order to explore disease-relevant BRD4 protein-protein interactions, genomic binding sites, and downstream transcriptional effects.

ABSTRACT

Human mast cells (MCs) are hematopoietic stem cell (HSC)-derived, tissue-resident, multifaceted cells involved in a myriad of physiological and pathological processes. Researches on MCs have been hampered for a long time, due to limited access to pure populations of these cells. Although some human MC lines were available, they all have limitations. For this reason, in a first part of our work, we have established a new human MC line, ROSA^{KIT WT}, whose properties are similar to those of primary HSC-derived MCs, providing a novel tool for research on human MC functions, and enabling the high-throughput screening of anti-allergic therapies. Among others, MCs are involved in a group of diseases termed mastocytosis, where they accumulate pathologically in various tissues. Although most cases of systemic mastocytosis (SM) are chronic with an indolent course, patients with advanced SM (aggressive SM; ASM, and mast cell leukemia; MCL) have a reduced life expectancy and a poor prognosis, since most of the therapies already available are not curative. In order to better understand the pathophysiology of advanced SM and to find new approaches for treatment, we took advantage of the availability of the ROSA^{KIT WT} cells to establish a new subclone, the ROSA^{KIT D816V} cell line, representing a paradigm of the neoplastic cells accumulating in SM. Using these malignant cell line and patients' cells, we have been able to identify the epigenetic reader bromodomain-containing protein-4 (BRD4) as a novel drug target in ASM and MCL. Indeed, we demonstrated that neoplastic MCs from ASM patients expressed substantial amounts of BRD4. Interestingly, we then demonstrated that HMC-1 and ROSA^{KIT D816V} cell lines express BRD4, and that their proliferation is inhibited by a BRD4-specific shRNA. Moreover, we showed that the BRD4-targeting drug JQ1 induced a dose-dependent growth inhibition and apoptosis in the same cells. In addition, we demonstrated that JQ1 suppressed also the proliferation of primary neoplastic MCs of patients with ASM or MCL at low concentrations. Finally, we reported that midostaurin (PKC412) and all-trans retinoic acid (ATRA) cooperated with JQ1 in producing synergistic inhibitory effects on the survival of HMC-1 and ROSA cells. Altogether, our data represent a significant advance over what was previously known on the involvement of BRD4 in mastocytosis and identify this epigenetic reader bromodomain-containing protein as a promising drug target in advanced SM.

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Synthèse en Français de la thèse de Doctorat d'Université de Mme Ghaith WEDEH

Ecole Doctorale de Cancérologie - Université Paris XI

Titre: Identification de BRD4 comme nouvelle cible thérapeutique dans le traitement des mastocytoses systémiques agressives (ASM) et des leucémies à mastocytes (MCL)

Avant propos:

Les mastocytes sont des cellules granuleuses découvertes en 1878 par Paul Ehrlich, et retrouvées dans tous les tissus de l'organisme, bien que certains tissus conjonctifs, comme la peau, en soient particulièrement riches. Les mastocytes dérivent des cellules souches hématopoïétiques non engagées présentes dans la moelle osseuse (Welle 1997). Ces cellules souches donnent naissance à des progéniteurs mastocytaires de phénotype CD34+, KIT+, CD13+, sous l'influence de cytokines, en particulier le stem cell factor (SCF), dont le récepteur spécifique, dénommé KIT, est un récepteur transmembranaire à activité tyrosine kinase (TK) intrinsèque. Fait intéressant, KIT est exprimé sur les cellules souches hématopoïétiques et sur toutes les cellules de la lignée mastocytaire quelque soit leur stade de maturation (Arock *et al.* 2008). Les progéniteurs mastocytaires circulent puis migrent vers les tissus, sous l'influence de différentes cytokines (dont le SCF) et de chimiokines (dont le SDF-1) (Arock *et al.* 1993; Wedemeyer *et al.* 2000). Dans les tissus, ces précurseurs subissent les étapes finales de leur maturation, et en particulier, l'acquisition de granules intracellulaires matures, toujours sous l'influence de différentes cytokines comme le SCF. L'identification des mastocytes matures repose ainsi essentiellement sur la mise en évidence d'un cytoplasme rempli de très nombreuses granulations métachromatiques après coloration par des colorants basiques tels que le bleu de toluidine, sur leur positivité pour la tryptase et sur leur expression membranaire de récepteurs de haute affinité aux IgE (FcεRI). Chez l'homme on distingue deux types de mastocytes en fonction de leur contenu en protéases. Les mastocytes n'exprimant que la tryptase (MC_T) sont essentiellement retrouvés dans les muqueuses (digestives, pulmonaires, etc...), tandis

que les mastocytes exprimant la tryptase, mais aussi la chymase et la carboxypeptidase (MC_{TC}) sont surtout présents dans les tissus conjonctifs (peau, péritoine, etc...) (Vliagoftis & Befus 2005; Metcalfe 2008).

Les mastocytes jouent un rôle important dans l'initiation des réponses immunitaires innées et adaptatives en produisant les médiateurs chimiques de l'inflammation, en particulier l'histamine, l'héparine, la sérotonine, la tryptase et les cytokines. De plus, ces cellules jouent un rôle néfaste en tant qu'initiateurs des réactions allergiques immunoglobine E (IgE)-dépendantes, dites réaction allergique de type I. En effet, durant la phase de sensibilisation à un allergène multivalent, les IgE spécifiques produites par les lymphocytes B se fixent aux récepteurs de haute affinité aux IgE (FcεRI) se trouvant à la surface des mastocytes. Lors de la réintroduction de l'allergène multivalent, celui-ci vient agréger les FcεRI à la surface des mastocytes en se fixant sur les fragments Fab des IgE fixées à la surface de ces cellules. Cette agrégation provoque une activation, la dégranulation très rapide des mastocytes et donc une libération immédiate des médiateurs chimiques (histamine) et des protéases contenus dans les granules. De façon plus retardée, au cours de la même réaction allergique, les mastocytes sont aussi capables de synthétiser de nombreux autres médiateurs comme des dérivés des prostaglandines et des leucotriènes, mais aussi de nombreuses cytokines et chimiokines à activité proinflammatoire et/ou vasoactive.

Outre son implication dans l'immunité innée et acquise, dans les phénomènes de cicatrisation, de fibrose, voire d'auto-immunité (Abraham & Arock 1998; Galli *et al.* 2005; Metcalfe 2008), le mastocyte est impliqué directement dans les mastocytoses. Ces pathologies rares sont des néoplasies myéloïdes caractérisées par une accumulation anormale de mastocytes dans divers organes. Les organes généralement atteints sont la moelle osseuse, la peau, le foie, et le tractus gastro-intestinal (Valent *et al.* 2003; Horny *et al.* 2007; Metcalfe 2008; Amon *et al.* 2010; Sokol *et al.* 2013). Chez les enfants, la forme la plus fréquente est habituellement confinée à la peau (mastocytose cutanée ou CM) et peut régresser spontanément à l'adolescence. Par contre, la plupart des patients adultes présentent une forme systémique (mastocytose systémique ou SM), avec atteinte, au minimum de la moelle osseuse et, très souvent, de la peau (urticaire pigmentaire). Les mastocytoses systémiques sont le plus souvent indolentes (ISM), et plus

rarement graves. Les formes graves comportent une variante agressive (ASM) et une variante leucémique, aussi appelée leucémie à mastocytes (MCL). Le pronostic des patients atteints d'ASM ou de MCL est médiocre. Un certain nombre de patients atteints de SM vont présenter en même temps une autre hémopathie maligne non mastocytaire associée, le plus souvent myéloïde (SM-AHNMD) (Horny *et al.* 2008; Stoecker & Wang 2012). Dans ces cas, le pronostic est à la fois fonction de celui de la mastocytose et de celui de l'hémopathie maligne associée (Pardanani *et al.* 2009; Arock & Valent 2010).

Les symptômes cliniques des mastocytoses sont en rapport avec, d'une part, l'infiltration des différents tissus ou organes par les mastocytes pathologiques et, d'une autre part, avec la libération exagérée de médiateurs par ces cellules néoplasiques.

La physiopathologie des mastocytoses est dominée par la présence de mutations activatrices et acquises dans la structure du gène *KIT*. L'anomalie la plus fréquemment rencontrée chez l'adulte est la mutation ponctuelle *KIT* D816V, qui est détectable chez la plupart des patients avec mastocytose systémique. Par contre, chez l'enfant, la mutation *KIT* D816V n'est rencontrée que chez seulement un tiers des patients atteints de mastocytose, tandis que les autres malades présentent soit des mutations différentes de *KIT* (40% des patients), soit n'ont pas d'anomalies de *KIT*. Lorsqu'il y a une mutation de *KIT*, elle conduit à l'autophosphorylation constitutive du récepteur en absence de son ligand, le SCF.

Les traitements actuels des mastocytoses sont essentiellement symptomatiques et aucune thérapeutique spécifique ne permet d'obtenir la guérison de ces pathologies. En effet, le traitement des mastocytoses a pour but d'inhiber la libération des médiateurs par les mastocytes et/ou neutraliser les effets délétères de ces médiateurs et d'inhiber la survie des mastocytes pathologiques. Le traitement des mastocytoses systémiques indolentes utilisent généralement des antagonistes des récepteurs de l'histamine. Dans les mastocytoses agressives et les leucémies à mastocytes, des traitements cytoréducteurs comme l'interféron-alpha ou la cladribine (2-CdA) et/ou des traitements ciblant *KIT* sont utilisés, comme le PKC412 (midostaurine). Malheureusement, le pronostic de ces patients reste médiocre, même si lorsqu'ils sont traités par de nouveaux agents ciblant *KIT*, ou par polychimiothérapie et/ou même par greffe de cellules souches hématopoïétiques.

Notre laboratoire s'intéresse depuis de nombreuses années aux agents pharmacologiques capables d'inhiber la survie des mastocytes malins, et en particulier, à l'identification de nouvelles cibles thérapeutiques. En effet, comme décrit précédemment, les traitements actuels des mastocytoses systémiques, en particulier dans les formes agressives, sont décevants et même la midostaurine (PKC412) présente un intérêt limité dans la mesure où elle ne permet pas la guérison définitive de ces patients. C'est pourquoi nous nous sommes intéressés aux protéines épigénétiques qui ont émergé récemment comme des cibles anticancéreuses nouvelles, et parmi celles-ci, plus particulièrement aux protéines BET (bromodomaine et domaine terminal) qui reconnaissent les histones lysine acétylée, et de cette façon régulent l'expression des gènes. En effet, il a été montré qu'une dérégulation de leur activité peut être responsable de diverses maladies, dont des pathologies tumorales.

Les protéines BET, qui sont multifonctionnelles, sont les membres d'une famille protéique extrêmement bien conservée au cours de l'évolution au sein de nombreux organismes. Chez les mammifères, la famille compte quatre membres, dont trois qui sont ubiquitaires et qui présentent des affinités différentes pour la chromatine, ce qui pourrait expliquer qu'elles ciblent des régions spécifiques du génome, à savoir BRD2, BRD3 et BRD4. Une autre protéine de cette famille, appelée BRDT est, elle, spécifique du tissu testiculaire (Shang *et al.* 2004; paillisson *et al.* 2007). La particularité de cette famille est que ses membres possèdent un double bromodomaine, leur permettant une association forte à la chromatine acétylée, sur différents résidus lysines acétylés (Florence & Faller 2001; Filippakopoulos *et al.* 2012; Belkina & Denis 2012). Les données de la littérature tendent à leur conférer une fonction importante dans le contrôle de la prolifération au sens large: régulation du cycle cellulaire, de la transcription et participation aux événements de divisions mitotiques et méiotiques. Elles semblent servir de «plate-forme fonctionnelle» et recruter des facteurs de transcription, voire les polymérases elles-mêmes, au niveau de régions d'euchromatine, permettant ainsi l'expression spécifique de gènes. Elles pourraient également participer à l'hérédité d'une mémoire épigénétique entre les cellules et jouer un rôle dans la maturation des cellules germinales. Ainsi, les protéines BET sont indispensables chez les différents organismes par leur rôle central potentiel d'intermédiaire entre la chromatine et les facteurs nucléaires. Contrairement aux

autres facteurs d'organisation de la chromatine, en particulier les autres protéines à bromodomaine, les protéines BET sont capables de rester fixées aux chromosomes durant la division mitotique. Ceci laisse supposer qu'elles puissent jouer un rôle important au cours de la mitose et qu'elles participent au maintien d'une mémoire épigénétique à travers les divisions cellulaires.

Les protéines BET possèdent également un second domaine caractérisé qui leur est propre, le domaine Extra-Terminal, permettant des interactions protéine-protéine (Florence & Faller 2001; Belkina & Denis 2012). Ainsi, cette famille de protéine est au carrefour de l'homéostasie cellulaire et des informations épigénétiques permettant la mise en place d'un programme génétique spécifique à la fonction d'une cellule.

Parmi les protéines BET, nous nous sommes intéressés plus particulièrement à la protéine BRD4. Cette protéine est capitale pour la machinerie transcriptionnelle, car par le biais de son domaine CTM (C-terminal motif), elle recrute, au niveau de régions de chromatine acétylée, le facteur P-TEFb, permettant alors l'activation de la phase d'élongation de la transcription (Wang & Filippakopoulos 2015). Par ailleurs, Les bromodomains de BRD4 sont capables, quant à eux, de reconnaître H3K14ac, H4K5ac et H4K12ac (Dey *et al.* 2003). Il est également intéressant de noter que BRD4 est capable d'interagir avec la sous-unité RelA du facteur de transcription NF- κ B acétylé sur la lysine 301, ce qui laisse supposer que les histones ne sont pas les seuls partenaires acétylés reconnus par les bromodomains (Huang *et al.* 2009).

Fait notable, il est bien établi que des translocations chromosomiques impliquant les gènes BRD3 ou BRD4 sont impliquées dans des cancers extrêmement agressifs (French *et al.* 2008). BRD2 est surexprimée dans des lymphomes et des lignées leucémiques, tandis que BRD1 est impliquée dans des cancers du poumon.

Par ailleurs, il a été montré que l'inhibition de BRD4 par des siRNA ou par les inhibiteurs de BET tels que le composé JQ1 induit la mort de cellules tumorales *in vitro* ainsi qu'*in vivo* dans divers cancers. Ainsi, de nombreuses publications ont rapporté le potentiel thérapeutique prometteur de ces inhibiteurs *in vitro* et *in vivo* dans plusieurs hémopathies malignes, comme le lymphome de Burkitt, le myélome

multiple ou les leucémies aiguës myéloïdes (LAM) (Zuber *et al.* 2011;.Dawson *et al.* 2011; Mertz *et al.* 2011;. Delmore *et al.* 2011;.Herrmann *et al.* 2013).

Objectifs de la thèse

Mon projet de recherche, au cours de ma thèse, a visé dans un premier temps à établir de nouvelles lignées mastocytaires humaines normales ou néoplasiques, à partir de cellules souches CD34+ de sang de cordon. Nous avons ainsi pu établir *in vitro* un modèle cellulaire qui représente morphologiquement et biologiquement les mastocytes humains normaux et un autre modèle cellulaire qui représente au mieux les mastocytes maligns retrouvés chez les patients atteints de mastocytoses avancées. Nous nous sommes ensuite intéressé, dans un second temps, à étudier le potentiel thérapeutique de l'inhibition du lecteur épigénétique BRD4 chez les patients atteints de mastocytoses systémiques (SM) avancées (ASM et MCL).

Introduction de la thèse :

L'introduction en anglais de ma thèse comprend les chapitres suivants :

1. Les mastocytes

1.1. Aperçu historique

1.2. Le développement des mastocytes

1.2.1. Le SCF et son effet sur le développement des mastocytes

1.2.2. Effet d'autres cytokines et d'autres molécules biologiques sur le développement des mastocytes chez diverses espèces

1.3. La morphologie le phénotype et l'hétérogénéité des mastocytes

1.3.1. KIT, le récepteur tyrosine kinase du SCF

1.3.2. Voies de signalisation du récepteur KIT

1.3.3. Les régulateurs de l'activité kinase KIT et de la signalisation KIT

1.3.4. Mutations 'gain de fonction' et mécanismes de l'activation constitutive de KIT

1.4. Le récepteur de haute affinité pour l'IgE (FcεRI)

1.4.1. Structure et expression de FcεRI

1.4.2. Régulation de l'expression FcεRI

1.4.3. Voies de signalisation de FcεRI

1.5. D'autres récepteurs mastocytaires

1.6. Médiateurs mastocytaires

1.7. Activateurs de mastocytes

1.8. Fonctions des mastocytes dans la physiologie et dans les situations pathologiques

1.8.1. Fonction des mastocytes dans la défense de l'hôte, l'immunité innée et acquise

1.8.2. Le rôle des mastocytes dans l'allergie, l'asthme, le remodelage tissulaire et la fibrose

1.8.3. Le rôle des mastocytes dans les maladies vasculaires

1.8.4. Le rôle des mastocytes dans l'auto-immunité et d'autres maladies

1.8.5. L'implication des mastocytes dans le cancer et d'autres situations pathologiques

1.9. Les lignées mastocytaires humaines

1.9.1. La lignée mastocytaire humaine HMC- 1 et ses deux sous-clones (HMC-1.1 et HMC-1.2)

1.9.2. La lignée LAD

1.9.3. La lignée LUVA

1.9.4. La lignée MCPV-1

2. Les mastocytoses

2.1. Définition, épidémiologie et diagnostic

2.2. Classification des mastocytoses

2.2.1. Mastocytose cutanée (CM)

2.2.2. Mastocytose systémique indolente (ISM)

2.2.3. Mastocytose systémique associée à une hémopathie clonale non mastocytaire (SM-AHNMD)

2.2.4. Mastocytose systémique agressive (ASM)

2.2.5. Leucémie à mastocytes (MCL)

2.2.6. Sarcome mastocytaire (MCS)

2.2.7. Mastocytome extra-cutané (ECM)

2.3. Les caractéristiques cliniques des mastocytoses

2.4. Physiopathologie des mastocytoses

2.5. Le traitement des mastocytoses

2.5.1. Traitement symptomatique de la mastocytose

2.5.2. Thérapie cytoréductrice non spécifique

2.5.2.1. Interféron-alpha (IFN- α)

2.5.2.2. 2-Chlorodésoxyadénosine (cladribine ou 2-CdA)

2.5.2.3. Hydroxyurée (HU)

2.5.3. De nouvelles approches dans le traitement de la mastocytose: thérapies ciblées

2.5.3.1. Imatinib

2.5.3.2. Masitinib

2.5.3.3. Dasatinib (BMS354825)

2.5.3.4. Midostaurine (PKC412)

2.5.3.5. Nilotinib (AMN107)

2.5.3.6. Ponatinib (AP24534)

2.5.3.7. Nouveaux ITK

2.5.4. La transplantation allogénique de cellules souches hématopoïétiques (allo- CSH)

2.5.5. Cibles thérapeutiques alternatives possibles et perspectives d'avenir

2.5.5.1. Les cibles liées à la signalisation ou l'apoptose

2.5.5.2. Ciblage d'antigène

3. Protéines à Bromodomaine de type 4 (BRD4)

3.1. Bromodomains (BRD): lecteurs de résidus acétyl lysine

3.2. Structure des bromodomains

3.3. La famille de protéines BET

3.3.1. Les membres de la famille de protéines BET

3.3.2. L'architecture du domaine BET

3.4. Fonctions biologiques de BRD4 et des protéines BET

3.4.1. Les protéines Brd4 et BET sont des marqueurs de mitose et des régulateurs du cycle cellulaire

3.4.2. Les protéines BET et BRD4 sont des protéines «échaffaudages».

3.4.3. Les protéines BET et BRD4 fonctionnent comme des régulateurs de transcription

3.5. Rôle des protéines BET dans le cancer

3.5.1. Rôle des protéines BET dans les tumeurs solides

3.5.2. Rôle des protéines BET dans les hémopathies malignes

3.5.3. Rôle des inhibiteurs de BET dans les hémopathies et les tumeurs solides

Résultats obtenus

Au cours de ma thèse, nous avons établi, à partir de cellules souches CD34+ de sang de cordon cultivées à long terme en présence de SCF, une nouvelle lignée mastocytaire humaine stable, dénommée ROSA^{KIT}^{WT}, exprimant à la fois le récepteur FcεRI et le récepteur KIT de structure normale. Cette lignée cellulaire, que nous avons caractérisée, reste dépendante du SCF pour sa survie et sa prolifération (Saleh, Wedeh *et al.* 2014). Fait intéressant, son temps de doublement est court, elle est aisément congelable et parfaitement activable par aggrégation de ses récepteurs de haute affinité aux IgE.

Pour pouvoir étudier le rôle transformant du principal mutant de *KIT* retrouvé au cours des mastocytoses systémiques, nous avons transfecté ces cellules ROSA^{KIT}^{WT} par un vecteur lentiviral apportant une construction codant pour le *KIT* muté en D816V. Ceci nous a permis d'établir une nouvelle lignée mastocytaire humaine SCF indépendante, dénommée ROSA^{KIT}^{D816V}. Nous avons ainsi pu effectuer diverses études sur le phénotype de la lignée ROSA^{KIT}^{D816V}, ainsi que sur la signalisation intracellulaire liée à la mutation activatrice de KIT (Saleh, Wedeh *et al.* 2014). Fait intéressant, nous avons pu montrer

que, après injection intraveineuse dans la veine de la queue des souris NOD/SCID IL-2R γ ^{-/-} (souris NSG), les cellules ROSA^{KIT D816V} peuvent greffer à un niveau élevé ces animaux, non seulement dans la moelle osseuse, mais également dans la rate et les poumons, donnant lieu à une maladie qui reproduit au mieux la situation rencontrée chez les patients atteints de mastocytose systémique agressive. En effet, comme en témoigne l'immunomarquage de tryptase humaine sur les biopsies de moelle osseuse de souris NSG injectées par voie intraveineuse avec des cellules ROSA^{KIT D816V}, des infiltrats compacts de mastocytes humains tryptase positifs peuvent être observés. Par ailleurs, nous avons observé que les cellules ROSA^{KIT D816V} présentes sur les frottis médullaires des souris greffées prennent un aspect fusiforme, comparable à ce qui est observé dans les prélèvements médullaires des patients atteints de mastocytose systémique. Ainsi, notre modèle animal présente plusieurs caractéristiques ressemblant à celles des mastocytoses systémiques chez l'homme. Par ailleurs, nous avons pu montrer qu'aussi bien les cellules ROSA^{KIT D816V} que les cellules néoplasiques de patients atteints de MCL ou d'ASM ne répondent pas à l'activation anti-IgE-dépendante, bien qu'elles expriment de façon significative le récepteur Fc ϵ RI.

Les deux lignées ROSA^{KIT WT} et ROSA^{KIT D816V} ont fait l'objet d'un brevet international publié (brevet WO/2013/064639) et d'une publication où je suis co-premier auteur (Saleh, Wedeh *et al.* 2014).

Par la suite, nous avons pu montrer par immunohistochimie que la protéine BRD4 est surexprimée dans le cytoplasme des mastocytes médullaires des patients atteints d'ASM ainsi que de MCL, alors que les mastocytes médullaires des patients atteints d'ISM n'expriment pas de BRD4 cytoplasmique. Par ailleurs, en utilisant une technique de PCR quantitative, nous avons aussi montré l'existence d'une surexpression significative d'ARN messagers codants pour BRD4 dans les cellules des patients atteints d'ASM ainsi que de MCL, par rapport à l'expression de ce même messenger dans des mastocytes normaux ou dans des cellules néoplasiques provenant de patients atteints la forme indolente de la maladie (Wedeh *et al.* 2015).

De plus, nous avons pu montrer que les lignées mastocytaires humaines, comme la lignée HMC-1.1 (lignée mastocytaire leucémique présentant une mutation activatrice de *KIT* V560G), la lignée HMC-1.2 (lignée mastocytaire leucémique présentant deux mutations activatrices de *KIT*: V560G et D816V), la

lignée ROSA^{KIT WT} et la lignée ROSA^{KIT D816V}, expriment des quantités importantes de la protéine BRD4 dans leur cytoplasme (Wedeh *et al.* 2015).

En outre, afin d'étudier les mécanismes contribuant à la surexpression de BRD4 et les voies de signalisation potentiellement impliquées dans cette expression anormale par les mastocytes néoplasiques, nous avons traité les lignées mastocytaires humaines avec des inhibiteurs de divers signaux de signalisation, comme des inhibiteurs de tyrosine kinase (ITK) tels que l'imatinib, le dasatinib, le PKC412 et le Ponatinib, des inhibiteurs de MEK, des inhibiteurs de la voie PI3-kinase/mTOR, mais aussi par de l'acide rétinoïque tout-trans (ATRA), de la cladribine (2-CdA), des agents déméthylants, des inhibiteurs de JAK2 ou des inhibiteurs de STAT5. Nous avons ainsi pu montrer que le PKC412 et le dasatinib, qui sont connus pour bloquer l'activité tyrosine kinase du mutant KIT D816V, peuvent réguler négativement l'expression de BRD4 dans les cellules HMC-1.1, HMC-1.2, ROSA^{KIT WT} et ROSA^{KIT D816V}. En outre, nous avons constaté que différents inhibiteurs de MEK et la voie PI3K/mTOR, comme le composé BEZ235, peuvent réguler négativement l'expression de BRD4 dans les mêmes lignées mastocytaires humaines. Comme MEK et la PI3-kinase sont des effecteurs en aval de KIT, nos données suggèrent que KIT D816V, mais aussi d'autres mutants de KIT et même le KIT WT, pourraient contribuer à l'expression de BRD4 dans les mastocytes néoplasiques. Cette conclusion est en accord avec l'observation que le SCF favorise l'expression de BRD4 dans les cellules ROSA^{KIT D816V}. En revanche le même effet du SCF n'a pas été observé dans les cellules HMC-1. Ces résultats suggèrent donc que l'expression de BRD4 dans les mastocytes malins dépend non seulement de l'état d'activation de KIT, mais aussi d'autres facteurs (Wedeh *et al.* 2015).

Comme il a été récemment montré que le composé JQ1, inhibiteur de BRD4, exerce de puissants effets anti-néoplastiques dans des cellules de leucémies aiguës myéloïdes résistantes aux chimiothérapies (Sotlar *et al.* 2003; Herrmann *et al.* 2012), nous nous sommes intéressés aux effets de cette molécule dans nos modèles. Nous avons pu montrer, en utilisant le composé JQ1 ou un shRNA spécifique pour BRD4, que l'inhibition de BRD4 conduit à l'arrêt de la prolifération de toutes les lignées mastocytaires humaines testées. Les effets de JQ1 sur la croissance des cellules mastocytaires malignes ont été démontrés comme

étant dose-dépendants. De plus, nous avons montré que le knock-down de BRD4 est associé à l'induction de l'apoptose et à la diminution d'ARN messagers codants pour MYC (Wedeh *et al.* 2015). Un même effet inducteur d'apoptose, dose-dépendant, a été observé avec le composé JQ1 dans toutes les lignées mastocytaires humaines testées (Wedeh *et al.* 2015). En outre, nous avons pu montrer que le composé JQ1 inhibe la progression dans le cycle cellulaire de toutes les lignées mastocytaires humaines testées.

Fait intéressant, nous avons aussi observé que l'effet de JQ1 sur BRD4 conduit à l'inhibition de la prolifération des mastocytes médullaires primaires obtenus à partir de prélèvements médullaires de patients atteints de la forme agressive des mastocytoses systémiques et ce, à une CI50 plus faible que celle observée pour les lignées mastocytaires. Ces données suggèrent que la protéine BRD4 joue un rôle important dans la prolifération et la survie des mastocytes malins *in vivo*. Néanmoins, les mécanismes d'action et les fonctions biochimiques de la protéine BRD4 présente dans le cytoplasme des mastocytes malins restent à l'heure actuelle inconnus.

Comme le composé JQ1 a été montré comme capable d'induire une maturation des cellules blastiques au cours des leucémies aiguës myéloïdes (Zuber *et al.* 2011; Herrmann *et al.* 2012), nous nous sommes donc demandés si la même molécule pourrait aussi induire la maturation des cellules HMC-1 et/ou des cellules ROSA. Nous n'avons pu détecter aucun effet de ce composé sur la maturation morphologique de ces cellules. De plus, nous avons pu démontrer que le composé JQ1 induit une diminution dose-dépendante de l'expression de certains antigènes de différenciation (CDs) à la surface des cellules HMC-1 et des cellules ROSA, y compris CD63 (LAMP-3) et CD71 (récepteur de la transferrine). En outre, nous avons pu constater que JQ1 régule négativement l'expression membranaire de FcεRI sur les cellules ROSA^{KIT}^{WT}. Ceci peut suggérer le fait que l'expression de FcεRI est régulée par certains mécanismes épigénétiques dans ces cellules. Notre observation est la première décrivant un effet de JQ1 sur l'expression du FcεRI, cette expression étant d'ailleurs pour la première fois démontrée comme étant régulée par un mécanisme épigénétique impliquant une molécule à bromodomaine dans les mastocytes humains normaux ou néoplasiques.

Nous avons également pu montrer que le composé JQ1 supprime non seulement la libération d'histamine IgE-dépendante dans les cellules ROSA^{KIT WT} mais aussi l'augmentation de CD63 et de CD203c induite par les anti-IgE dans ces mêmes cellules, et ceci de façon dose-dépendante. Cette inhibition est probablement en rapport avec la régulation négative de l'expression membranaire du FcεRI sur ces cellules. Nous nous sommes ensuite demandés si le traitement des cellules ROSA^{KIT WT} avec des inhibiteurs de protéasome pourrait contrecarrer la régulation négative du FcεRI induite par le composé JQ1. Cependant, si aucun effet de ces inhibiteurs du protéasome n'a été trouvé dans ce cadre, nous avons observé que ces molécules exercent des effets antiprolifératifs sur ces cellules, avec des CI50 de l'ordre de 5 nM. Enfin, ces effets antiprolifératifs ont été associés à une induction d'apoptose non seulement dans les cellules ROSA^{KIT WT} mais également dans les cellules de ROSA^{KIT D816V}.

Par ailleurs, nous avons montré que l'expression de la tryptase dans les cellules ROSA^{KIT WT} diminue après traitement avec le composé JQ1 (Wedeh *et al.* 2015). En outre, JQ1 s'est avérée capable d'abroger l'expression de p-STAT3 dans les cellules ROSA^{KIT WT} et aussi de diminuer l'expression de p-STAT5 dans les cellules ROSA^{KIT D816V}, HMC-1.1 et HMC-1.2, et ce d'une manière dose-dépendante. Ces observations sont en cohérence avec une publication récente dans laquelle les auteurs ont rapporté que le composé JQ1 est capable d'inhiber l'expression de p-STAT5 dans les cellules d'hémopathies myéloïdes malignes (Liu *et al.* 2014). Prises ensemble, nos données suggèrent que JQ1 peut inhiber plusieurs voies moléculaires impliquées dans l'activation et la prolifération des mastocytes malins.

Par ailleurs, l'analyse par western-blot des voies de signalisation, menée sur les cellules HMC-1.1, HMC-1.2, ROSA^{KIT WT} et ROSA^{KIT D816V} lors du traitement par JQ1 nous a conduit à conclure que la voie MEK et celle de la PI3-Kinase sont impliquées dans le mécanisme sous-jacent de l'effet de JQ1 dans ces cellules. De ce fait, on peut envisager l'hypothèse que la voie de signalisation RAS pourrait être une des voies responsables des effets du composé JQ1.

Les effets importants de ce composé sur la croissance et la survie des mastocytes néoplasiques nous ont ainsi incités à rechercher des molécules à activité pharmacologique avec lesquels des effets synergiques antinéoplasiques pourraient être observés lorsqu'ils sont utilisés en combinaison avec JQ1. Dans ce but,

nous avons testé l'effet de différentes combinaisons de molécules sur les cellules HMC-1 et ROSA. De tous les médicaments testés, seuls le PKC412 et l'ATRA se sont montrés capables d'exercer une synergie avec JQ1 et d'inhiber la prolifération et la survie des cellules HMC-1 et ROSA de manière dose-dépendante en synergie avec ce dernier composé. Cette mise en évidence d'une synergie entre le PKC412 ou l'ATRA et le composé JQ1 pourrait avoir des implications en termes de thérapeutique des mastocytoses systémique agressives.

Dans une dernière étape de notre travail, nous nous sommes demandés si les mastocytes néoplasiques pourraient devenir résistants au composé JQ1 du fait de la présence de stem cell factor. En effet, la résistance aux thérapeutiques liée à un effet inhibiteur des cytokines est un problème majeur du traitement des hémopathies malignes. Ainsi, nous avons pu observer que le SCF réduit l'effet antiprolifératif du composé JQ1 sur les cellules ROSA^{KIT D816V} mais de façon un peu étonnante, pas sur les cellules HMC-1. Nous nous sommes alors demandés si l'utilisation de combinaisons JQ1 + ATRA ou JQ1 + PKC412 permettrait de surmonter cette résistance induite par le SCF dans les mastocytes néoplasiques. Ainsi, nous avons pu montrer que la combinaison JQ1 + PKC412 permet de surmonter cette résistance cytokine-dépendante des cellules ROSA^{KIT D816V}, tandis que l'utilisation de la combinaison JQ1 + ATRA n'a pas permis d'observer cet effet. Cette observation pourrait être expliquée par le fait que la résistance induite par le SCF est médiée par l'activation de KIT, et que donc ce type de résistance ne peut être surmonté que par l'ajout d'un agent inhibant de façon significative l'activité tyrosine kinase de KIT, ce qui est le cas du PKC412 (Wedeh *et al.* 2015).

En conclusion, notre travail est le premier à démontrer que BRD4 peut constituer une cible thérapeutique intéressante dans les mastocytoses systémiques graves (MCL et ASM). Les données que nous avons obtenues montrent également que l'inhibition de BRD4 par le composé JQ1 est une approche thérapeutique novatrice potentiellement capable d'éradiquer les mastocytes néoplasiques et que, de plus, les effets de cette molécule peuvent être potentialisés par l'addition de PKC412 ou d'ATRA.

Principaux articles publiés durant la thèse:

1. **Wedeh G**, Cerny-Reiterer S, Eisenwort G, Herrmann H, Blatt K, Hadzijusufovic E, Sadovnik I, Müllauer L, Schwaab J, Hoffmann T, Bradner JE, Radia D, Sperr WR, Hoermann G, Reiter A, Horny HP, Zuber J, Arock M, Valent P. Leukemia. 2015 Nov; 29(11): 2230-7
2. Saleh R*, **Wedeh G***, Herrmann H, Bibi S, Cerny-Reiterer S, Sadovnik I, Blatt K, Hadzijusufovic E, Jeanningros S, Blanc C, Legarff-Tavernier M, Chapiro E, Nguyen-Khac F, Subra F, Bonnemye P, Dubreuil P, Desplat V, Merle-Béral H, Willmann M, Rüllicke T, Valent P, Arock M. A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection. Blood. 2014 Jul;124(1): 111-20
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3. Akil A, **Wedeh G**, Zahid Mustafa M, Gassama-Diagne A. SUMO1 depletion prevents lipid droplet accumulation and HCV replication. Arch Virol. 2015 Oct 8. [Epub ahead of print]

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ANNEXES
Patents and certificates


Traduction automatique

1. (WO2013064639) LIGNEES DE MASTOCYTES HUMAINS, PREPARATION ET UTILISATIONS

Données bibliographiques PCT | Description | Revendications | Phase nationale | Notifications | Drawings | Documents

Dernières données bibliographiques dont dispose le Bureau international

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Données relatives à la priorité: 1159902 02.11.2011 FR

Titre

(EN) HUMAN MAST CELL LINES, PREPARATION AND USES
(FR) LIGNEES DE MASTOCYTES HUMAINS, PREPARATION ET UTILISATIONS

Abrégé:

(EN)The present invention relates to a human mast cell line corresponding to deposit number CNCM I-4551 and also to the lines derived therefrom, in particular the derived lines corresponding, respectively, to deposit numbers CNCM I-4552 and CNCM I-4553, and to the uses thereof, in particular for screening for compounds of therapeutic interest.
(FR)La présente invention concerne une lignée cellulaire mastocytaire humaine correspondant au numéro de dépôt CNCM I-4551 ainsi que les lignées dérivées de celle-ci, en particulier les lignées dérivées correspondant respectivement aux numéros de dépôt CNCM I-4552 et CNCM I-4553, et leurs utilisations, en particulier pour le criblage de composés d'intérêt thérapeutique.



États désignés:

AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
 Organisation régionale africaine de la propriété intellectuelle (ARIPO) (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW)
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Diploma Certificate

In recognition of the successful completion of the requisites and on nomination of the EuroCST Scientific Board, on the 26th of November 2014 by virtue of their authority, hereby confers upon GHAITH WEDEH the Diploma Certificate with all the honors, rights, and privileges thereto pertaining.



Ghaith Wedeh - Graduate

Professor Peter Valent - Supervisor

Professor Christine Chomienne - Coordinator

Doctor Hervé Chneiweiss - Tutor

ANNEXES

Publication on another topic issued during the thesis

"SUMO1 depletion prevents lipid droplet accumulation and HCV replication"

A Akil, G Wedeh, MZ Mustafa, A Gassama-Diagne. Arch Virol. 2016 Jan;161(1):141-8.

This work has been realized in collaboration with INSERM, UMR-S 785, 94800 at Villejuif, France.

SUMO1 depletion prevents lipid droplet accumulation and HCV replication

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Abstract Infection by hepatitis C virus (HCV) is a major public-health problem. Chronic infection often leads to cirrhosis, steatosis, and hepatocellular carcinoma. The life cycle of HCV depends on the host cell machinery and involves intimate interaction between viral and host proteins. However, the role of host proteins in the life cycle of HCV remains poorly understood. Here, we identify the small ubiquitin-related modifier (SUMO1) as a key host factor required for HCV replication. We performed a series of cell biology and biochemistry experiments using the HCV JFH-1 (Japanese fulminate hepatitis 1) genotype 2a strain, which produces infectious particles and recapitulates all the steps of the HCV life cycle. We observed that SUMO1 is upregulated in Huh7.5 infected cells. Reciprocally, SUMO1 was found to regulate the expression of viral core protein. Moreover, knockdown of SUMO1 using specific siRNA influenced the accumulation of lipid droplets and reduced HCV replication as measured by qRT-PCR. Thus, we identify SUMO1 as a key host factor required for HCV replication. To our knowledge, this is the

first report showing that SUMO1 regulates lipid droplets in the context of viral infection. Our report provides a meaningful insight into how HCV replicates and interacts with host proteins and is of significant importance for the field of HCV and RNA viruses.

Keywords HCV · SUMO1 · Lipid droplets

Introduction

Hepatitis C virus (HCV) has long been considered a public-health problem, with an estimated 170 million infected individuals worldwide. It is a major human pathogen responsible for development of serious liver diseases, including steatosis, fibrosis, cirrhosis, and eventually, hepatocellular carcinoma [1]. HCV is a small, enveloped, positive-sense, single-stranded RNA virus of the family *Flaviviridae* [2]. The HCV genome is transported to the endoplasmic reticulum, where it is translated into a large polyprotein by the host translation machinery and processed by viral and cellular proteases into the mature structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A-B, NS5A-B) [2–4]. HCV replication occurs in replication complexes (RCs) in a membranous web (MW) consisting of lipid droplets (LDs) [4, 5].

There is not yet a vaccine to protect against HCV infection. However, a major improvement regarding treatment of HCV infection has been recently achieved. Two direct-acting antivirals (DAAs), the protease inhibitors telaprevir and boceprevir, were approved for the treatment of HCV infection in combination with pegylated interferon alfa/ribavirin (peg-IFN/RBV) [6, 7]. The addition of DAAs targeting different viral proteins was found to increase the sustained virologic response (SVR) [8], defined as the lack

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of detection of HCV RNA in blood several months after a complete course of treatment, and therefore to improve treatment success [9–11]. Unfortunately, resistance to DAAs has been reported recently, due to the high genetic variability of the HCV RNA genome [12–14]. Therapies that target host cell pathways may overcome or limit the occurrence of viral resistance and thus improve treatment responses. In this therapeutic context, there is an increased need to define the pathways that are hijacked by HCV for its life cycle. Moreover, HCV infection is tightly associated with alterations in lipid metabolism, and lipids have been shown to play important roles during the viral life cycle [15, 16]. The HCV core induces the redistribution of LDs through the clustering of these organelles in the perinuclear area, providing a platform for virus replication, assembly and production [17].

LDs have long been considered simple reservoirs for lipid storage, but they are now considered dynamic organelles that are involved in many biological processes, and several reports have highlighted their movement by attachment to microtubules [15–19]. Nevertheless, the molecular details of the mechanisms driving LDs biogenesis, growth and intracellular movement remain largely unknown. Modification of proteins involved in lipogenesis, such as the sterol regulatory element-binding proteins (SREBPs) and liver receptor homolog 1 (LRH-1) by SUMO has been demonstrated [20, 21]. Additionally, Talamillo and coworkers have demonstrated that silencing the *Drosophila* SUMO homologue *smt3* in the prothoracic gland leads to reduced lipid content [23]. These recent advances suggest that SUMO might have critical roles in lipogenesis [20, 21, 23].

The SUMO protein was first identified in mammals, where it was found to be covalently linked to the GTPase-activating protein RanGAP1 [24]. Consequently, SUMO is expressed by all eukaryotes but is absent from prokaryotes/archaea. Lower eukaryotes have a single SUMO gene, whereas plants and vertebrates express several SUMO paralogues [25]. In mammals, the SUMO family consists of four different isoforms, termed SUMO1 to 4. The SUMOs are post-translationally conjugated to other proteins, thereby regulating diverse cellular processes, including transcription, DNA repair, chromosome dynamics, subcellular localization of proteins, and protein-protein interactions via a mechanism essentially analogous to that of ubiquitination [26, 27].

In addition to its critical roles in a variety of cellular signalling pathways, SUMO family members play important roles in the life cycle of various viruses, such as KSHV (Kaposi's sarcoma-associated virus) [28] and influenza virus [29]. In this study, we performed different experiments using HCVcc-JFH1 and investigated the importance of SUMO1 for HCV replication.

Materials and methods

Antibodies and reagents

Anti-mouse actin and oleic acid were obtained from Sigma, and anti-mouse core and anti-rabbit SUMO1 were from Abcam. Anti-mouse Alexa Fluor 488 and 546 were obtained from Invitrogen. Nuclei were stained with TO-PRO-3 Iodide or Hoechst from Invitrogen.

Cell culture

Huh7.5 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 4.5 g of glucose per liter, supplemented with 10 % heat-inactivated fetal bovine serum, 1 % nonessential amino acids (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen).

Small interfering RNA and cell transfection

Endogenous expression of SUMO1 was silenced by transfection with a specific siRNA (sc-29498, Santa Cruz). Huh7.5 cells were grown in 12-well plates or on glass coverslips and infected with HCVcc. At 24 hours post-infection, cells were transiently transfected with 100 pmol of a siRNA oligonucleotide against SUMO1 using a Lipofectamine RNAiMAX Kit (Invitrogen) according to the manufacturer's protocol and incubated for another 48 hours. Control non-targeting siRNA was used as a reference (Genecust). At 48 hours post-transfection, cells were harvested for extraction of proteins and isolation of total RNA or fixed with a 3.7 % paraformaldehyde. The samples were then analyzed via western blot, qRT-PCR and immunofluorescence.

Immunoblot

Cells were collected on ice, washed and lysed in 20 mM Tris-HCl, 100 mM NaCl, 1 % Triton X-100 and 10 mM EDTA, pH 7.4, containing protease and phosphatase inhibitor cocktail (Roche diagnostics). The proteins were separated by SDS-PAGE, blotted onto a PVDF membrane, and visualized using a chemiluminescence reagent (Amersham).

Immunofluorescence staining

Huh7.5 cells were grown on glass coverslips in 12-well plates, treated according to the experiments, fixed with 3.7 % paraformaldehyde, permeabilized, and saturated with PBS supplemented with 0.7 % fish gelatin and 0.025 % saponin. Primary antibodies were diluted with permeabilization solution and incubated with cells. The

cells were washed and stained with fluorescent secondary antibodies, phalloidin and TO-PRO 3 or Hoechst. Samples were observed with a Zeiss 510 LSM confocal microscope or a Leica TCS SP5 (Leica Microsystems). The lipophilic fluorescence dye LD 540 was used to stain lipid droplets.

JFH1/HCVcc production and inoculation

The JFH1/HCVcc stock was prepared as described [30]. Cells were infected with HCV at 37 °C for 16 h, and unbound viruses were removed by aspiration and by washing cells with PBS and DMEM. The infected cells were harvested for extraction of proteins and isolation of total RNA or fixed with a 3.7 % paraformaldehyde. The samples were then analyzed via western blot, qRT-PCR and immunofluorescence.

RNA extraction and qRT-PCR analysis

Total RNA was isolated using RNable solution (Eurobio), and intracellular levels of positive-strand HCV RNA were quantified by a strand-specific qRT-PCR technique described previously [36] using a Light Cycler Fast Start DNA MasterPlus SYBR Green (Roche) on a Real-Time PCR System (Roche). The triplicate mean values were calculated according to the Ct quantification method (Pfaffl 2001) using GAPDH gene transcription as reference for normalization. The average levels of HCV RNA relative to the positive control (100 %) from triplicate experiments, are shown.

Statistical analysis

Comparison of mean values was conducted using an unpaired Student's *t*-test. Statistical significance was determined at **P* < 0.05, ***P* < 0.001, and ****P* < 0.0001.

Results

The abundance of SUMO1 is altered in infected Huh7.5 cells

We examined the expression of SUMO1 in uninfected Huh7.5 cells (Mock) and infected Huh7.5 cells (HCV) (Fig. 1). Based on the specific bands size, immunoblot analysis indicated an increase in the amount of SUMO1 (Fig. 1B and Fig. 1C). In addition to investigating protein level of SUMO1, we examined the localization of SUMO-1 in uninfected and infected cells. SUMO-1 appeared in the nucleus and cytoplasm of uninfected and infected cells (Fig. 1A).

SUMO1 regulates HCV genomic RNA replication and core expression

We assessed the requirement for SUMO1 in HCV viral protein expression and RNA replication. First, Huh7.5 cells were infected with HCV-JFH1 for 24 hours and then treated with SUMO1 siRNA for another 48 hours and analyzed by immunoblot, immunofluorescence staining and qRT-PCR. A reduction in the SUMO1 signal was observed, and this was associated with a decrease in the core protein level (Fig. 1E). Next, the cells were analyzed by confocal microscopy. Treatment of cells with SUMO1 siRNA reduced cellular expression of the core protein, as shown by immunoblotting and immunofluorescence (Fig. 1D). After that, we tested the effect of SUMO1 on HCV replication using qRT-PCR. The intracellular level of HCV RNA was quantified, and a significant drop in the level of HCV genomic RNA was observed after depletion of SUMO1 (Fig. 1F), indicating a critical role for this protein in HCV replication. The efficiency of SUMO1 siRNA was evaluated by immunoblotting and immunofluorescence staining (Fig. 1E and D).

Depletion of SUMO1 affects colocalization of HCV core protein and LDs

Attachment of the core protein to LDs is crucial for HCV replication [15]. Recent advances suggest that SUMO might have critical roles in lipogenesis [20–22], silencing the drosophila SUMO in the prothoracic gland has been shown to result in reduced lipid content [23]. Taking these data into account, we analysed the behaviour of LDs in SUMO1-siRNA-treated infected Huh7.5 cells. As presented in Fig. 2A, in uninfected cells, LDs were present at low levels and dispersed in the cytoplasm, whereas in infected cells, there was an increase and redistribution of LDs as clusters that colocalized with core protein around the nucleus (Fig. 2B and SI Fig. 1A). Treatment of cells with SUMO1 siRNA strongly reduced accumulation of LDs and cluster formation (Fig. 2C). Confocal images were analysed using Image J software, and the results are presented as graph under to each panel with a cartoon representing the LD content of the cell separated in three zones from the nucleus (Z1, Z2, and Z3). The LD signal was mainly present around the nucleus (Z2), and the intensity of the LD signal strongly increased with HCV infection. However, treatment of cells with SUMO1 siRNA led to a decrease in the intensity of the LD signal and a shift from the centre to the cell periphery. Fifty cells were analysed from three experiments, and the intensity of the LD signal was evaluated using Image J software. The data are presented as histograms in Fig. 2C and D. The number of LDs and the percentage of cells expressing LDs

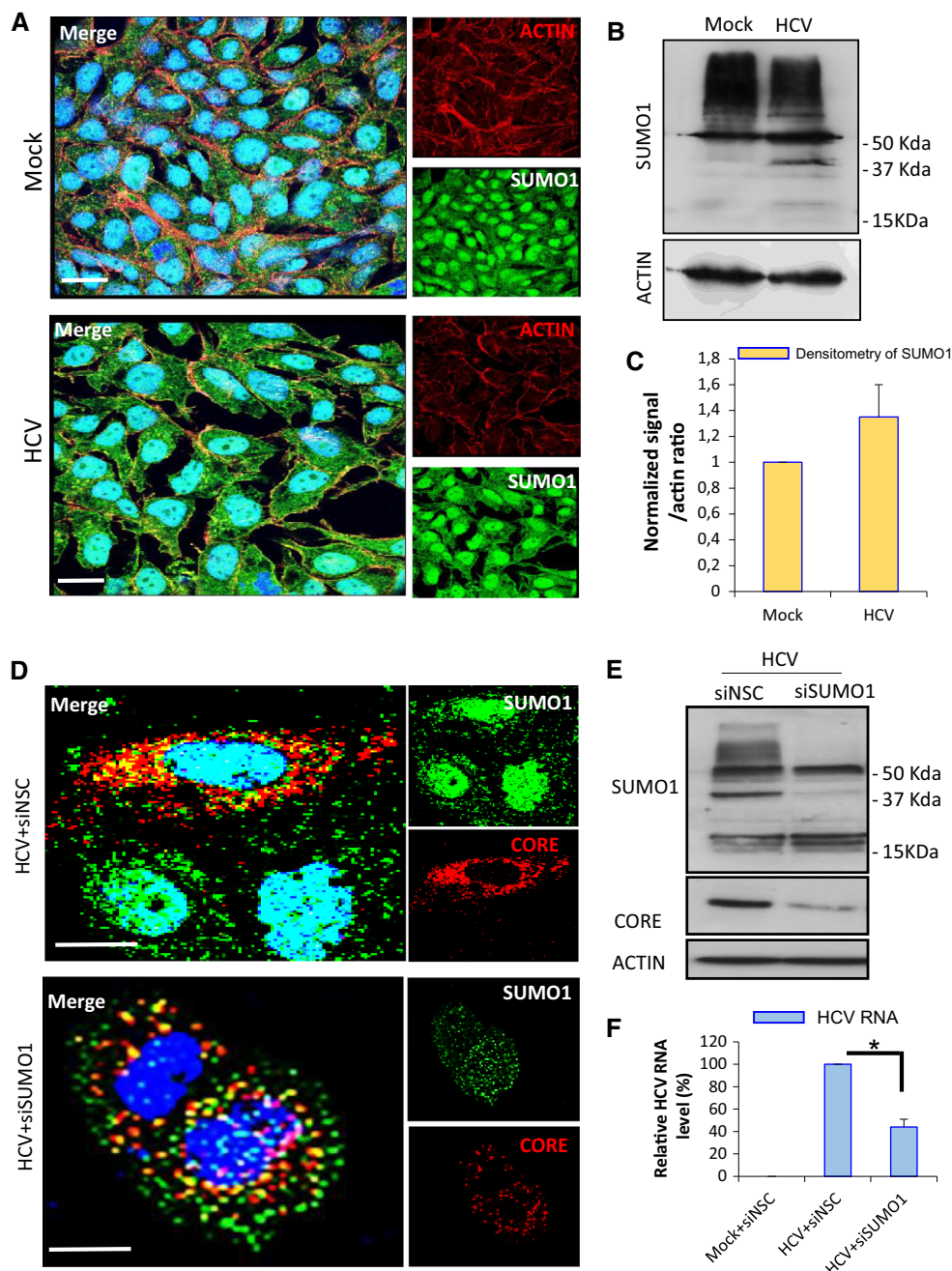


Fig. 1 SUMO1 regulates expression of the HCV core protein and genomic RNA expression. (A) Immunofluorescence analysis of SUMO1 (green), actin (red) and nuclei (blue) in uninfected (Mock) and infected (HCV) Huh7.5 cells. Scale bars represent 10 μ m). (B) Immunoblot analysis of SUMO1 and HCV core in lysates of uninfected and infected Huh7.5 cells. Actin was used as a loading control. (C) Data analysis of the immunoblot staining in B, recapitulated as a histogram. (D) Immunofluorescence analysis of SUMO1 (green), core (red) and nuclei (blue) in Huh7.5 cells infected with HCV for 24 hours and then transfected with control siRNA (siNSC) or with SUMO1 siRNA (100 pmoles/assay) at 37 $^{\circ}$ C. At 48 hours post-transfection, cells were fixed with 3.7 % paraformaldehyde and stained as described in Materials and methods. Scale bars, 10 μ m. (E) Immunoblot analysis of HCV core and SUMO1 in Huh7.5

cells infected with HCV for 24 hours and then transfected with control siRNA (siNSC) or with SUMO1 siRNAs (100 pmoles/assay) at 37 $^{\circ}$ C. At 48 hours post-transfection, cells were harvested for protein extraction. Actin was used as loading control. (F) Inhibition of HCV replication in Huh7.5 cells by siRNA specific for SUMO1. Huh7.5 cells in 12-well plates were infected with HCV. At 24 hours postinfection, cells were transiently transfected with control siRNA (siNSC) or with SUMO1 siRNA and incubated for another 48 hours at 37 $^{\circ}$ C. Total RNAs in the uninfected and infected Huh7.5 cells were isolated with RNABLE reagent (Eurobio). The levels of positive-strand HCV RNA were determined by qRT-PCR as described in Materials and methods. The average levels of HCV RNA relative to the control (100 %), from triplicate experiments, are shown (color figure online)

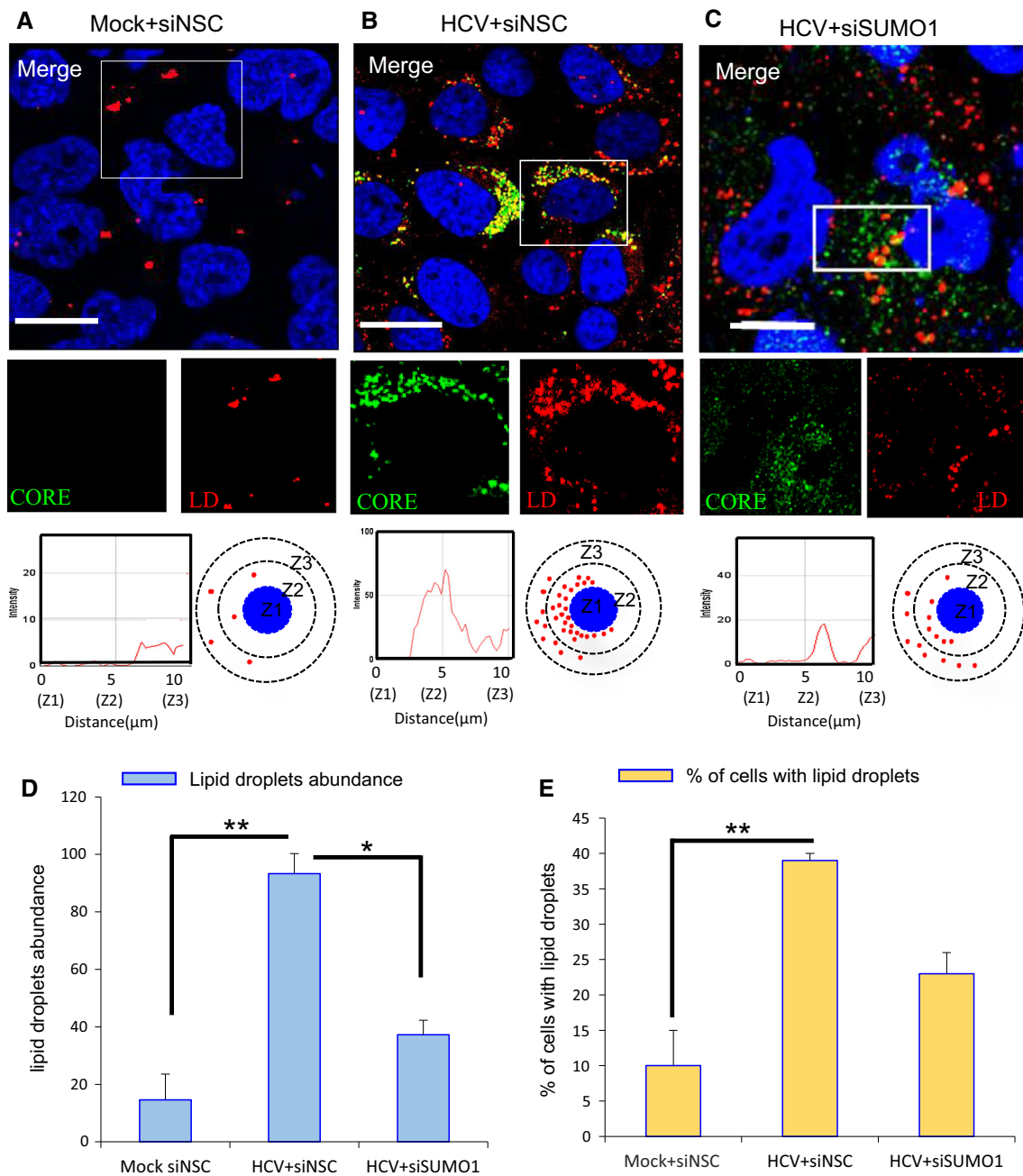


Fig. 2 Effect of SUMO1 on LD formation and on their association with the HCV core protein. (A) Immunofluorescence labeling of lipid droplets (red), core (green) and nuclei (blue) in uninfected Huh7.5 cells (Mock) treated with control siRNA. Scale bar, 10 μ m. (B) Immunofluorescence labeling of lipid droplets (red), core (green) and nuclei (blue) in infected Huh7.5 cells treated with control siRNA as in Fig. 1D. Scale bar, 10 μ m. (C) Immunofluorescence labeling of lipid

droplets (red), core (green) and nuclei (blue) in infected Huh7.5 cells treated with SUMO1 siRNA as in Fig. 1D. Scale bar, 10 μ m. (D) The average number of lipid droplets according to the experiments in panels A, B and C (lipid droplet count). (E) The effect of SUMO1 on the percentage of cells that expressed lipid droplets (color figure online)

increased in the presence of HCV infection (Fig. 2D). However, the silencing of SUMO1 expression led to a statistically significant decrease in the number of LDs (Fig. 2D) and also caused a reduction in the percentage of cells expressing LDs (Fig. 2E), indicating a role of SUMO1 in HCV induced- accumulation of LDs.

Depletion of SUMO1 alters the cellular level and distribution of LDs

Because of the important regulatory function of SUMO1 on LD accumulation, we decided to analyse the effect of SUMO1 on LD accumulation induced by oleic acid

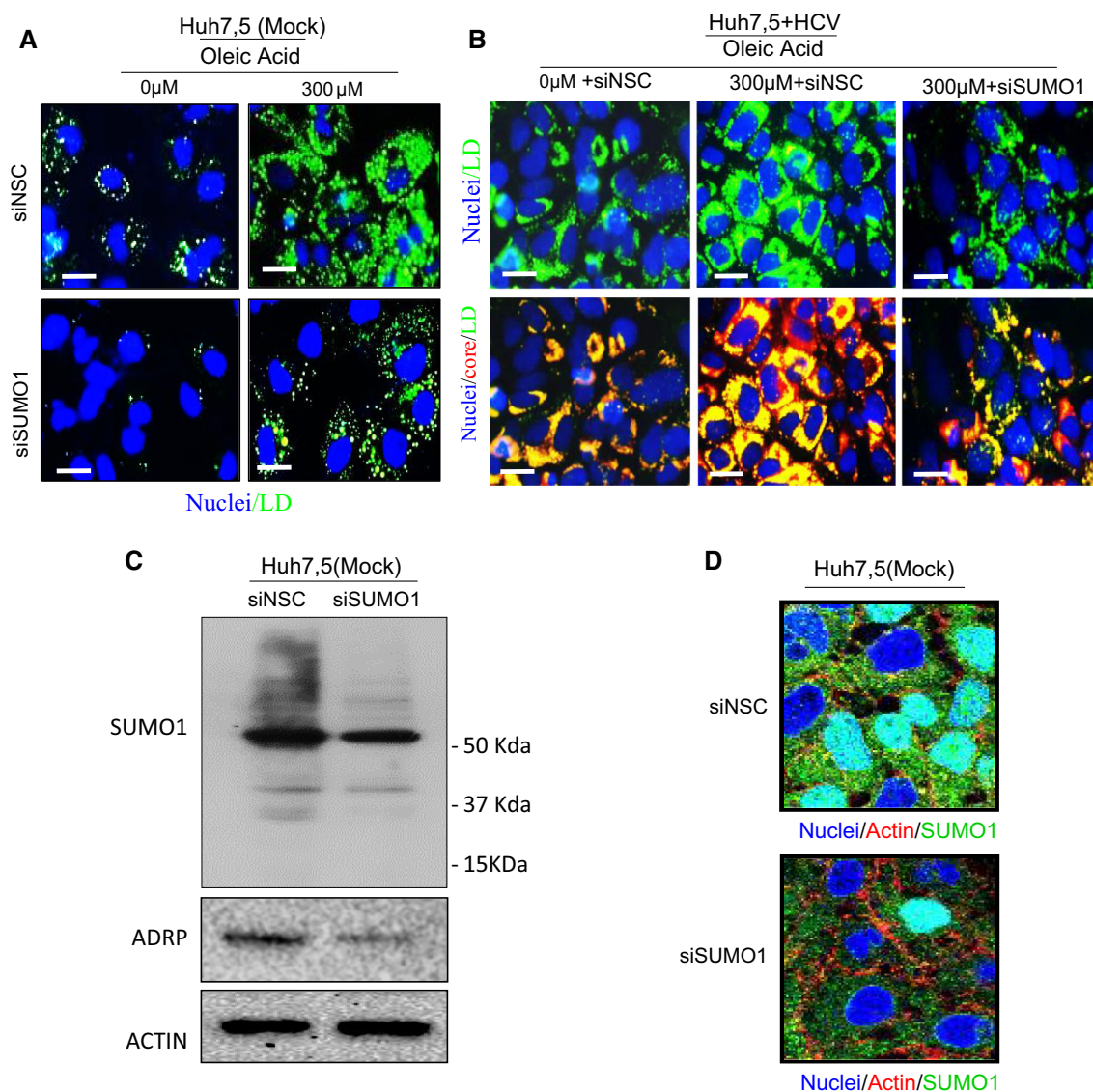


Fig. 3 SUMO1 alters cellular accumulation of LDs. (A) Immunofluorescence analysis of LD (green) and nuclei (blue) in uninfected cells treated with 300 μ M oleic acid for 24 hours and then transfected with control siRNA or SUMO1 siRNA (100 pmoles/assay) for another 48 hours. Scale bar, 10 μ m. (B) Immunofluorescence analysis of LD (green) and nuclei (blue) and core (red) in infected cells treated with 300 μ M of oleic acid for 24 hours, transfected with control siRNA or

(Fig. 3A and B). Interestingly, treatment of cells with siRNA against SUMO1 suppressed LD formation induced by oleic acid in uninfected (Fig. 3A) and in infected cells (Fig. 3B), indicating that SUMO1 is required for formation of lipid droplets. Moreover, depletion of SUMO1 affected the expression of adipose differentiation-related protein (ADRP), an important protein associated with lipid droplets (Fig. 3C), confirming that knockdown of SUMO1 leads to reduced lipid content. The efficiency of SUMO1 siRNA was evaluated by immunoblot and immunofluorescence staining (Fig. 3C and D).

SUMO1 siRNA (100 pmoles/assay). Scale bar, 10 μ m. (D) Immunoblot analysis of SUMO1 and ADRP in lysates of uninfected Huh7.5 cells treated with control or with SUMO1 siRNAs for 48 hours. (E) Immunofluorescence analysis of SUMO1 and actin in uninfected Huh7.5 cells treated as in Fig. 3D. Scale bar, 10 μ m (color figure online)

Discussion

This study provides another example of SUMO being needed for viral infection. We showed that HCV infection increases SUMO1 expression, which is necessary for its replication. This work provided significant insights into the host cellular machineries exploited by HCV for its replication, as well as new fundamental data on the functional features of SUMO1.

Different studies have suggested that LDs play a crucial role in the life cycle of HCV. Interactions between HCV

proteins, especially the core protein and LDs, are required for the morphogenesis and production of infectious HCV [31]. Our study revealed that SUMO1 modulated the accumulation of LDs and their association with HCV core and thus contributed to the establishment of the lipid environment required for virus replication.

SUMO proteins are important regulators of cell signalling and are covalently linked to other proteins in order to alter their structure, localization or function. To date, descriptions of SUMO conjugated pathogen proteins are limited to viruses, for which protein SUMOylation facilitates viral entry, nuclear translocation, changes in gene expression, and vesicular trafficking [34]. Studies conducted by Dunphy and coworkers have shown that *Ehrlichia chaffeensis* exploits SUMOylation pathways to promote its intracellular survival [33]. Moreover, previous work has shown that global cellular SUMOylation increases during influenza virus infection, and silencing the key enzymes required for SUMO conjugation prevents virus replication [32]. Our own work indicated also that negative changes in SUMO1 levels disturb HCV replication. This raised the possibility that this virus may manipulate SUMO and/or the fate of SUMO-modified proteins to facilitate its replication. In this report, SUMO1 was found mainly within the nucleus in both uninfected and infected cells. However, there appeared to be an increase in SUMOylation during HCV infection. This change in SUMO1 might be an important step for determining the abundance of lipid droplets and for their recruitment, a process that is necessary for HCV replication. It is difficult to provide a simple hypothesis that attempts to explain these results, because the biological effects of SUMOylation are quite diverse, and the mechanisms and signal pathways involved in most of them remain unclear. Recent advances suggest that SUMO might have critical roles in lipogenesis [20–22]. Moreover, silencing of SUMO in the prothoracic gland of drosophila led to reduced lipid content [23]. More experiments will be needed to identify the factors involved in the connection of SUMO1 and lipid droplets. This study provided information about the role of SUMO1 in production and redistribution of LDs, which is timely, because there is increasing evidence in the literature that lipid droplets play a crucial role in the replication of different pathogens [35]. Our study suggested that HCV may alter the localization and abundance of SUMO1, and this, in turn, might help to recruit and organize the LDs required for HCV assembly and replication. Thus, one explanation for the implication of SUMO1 in LDs redistribution could be connected to the SUMO E3 ligase function.

To conclude, this study illuminates an important function for SUMO1 in HCV replication. These findings also represent a step forward in our understanding of the

relationship between HCV and its host and may open new therapeutic directions. A future key aim may be to correlate changes to the SUMO sub-proteome with the function of lipid droplet biogenesis, thereby establishing the mechanistic role of these modifications during HCV replication. Understanding the basic mechanisms underlying SUMOylation in HCV infection will provide new insights into the fundamental biology of this important pathogen. Future studies focused on the key enzymes required for general SUMOylation could also lead to identification of key cellular pathways that can be exploited by novel therapeutic targets.

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Titre: Identification de BRD4 comme nouvelle cible thérapeutique dans le traitement des mastocytoses systémiques agressives (ASM) et des leucémies à mastocytes (MCL)

Mots clés: BRD4, mastocytoses systémiques agressives, leucémies à mastocytes, cible thérapeutique

Résumé: Les mastocytes humains (MC) sont des cellules tissulaires d'origine hématopoïétique impliquées dans une série de processus physiologiques et pathologiques. Les recherches sur les MC ont été entravées pendant longtemps en raison de l'accès limité à des populations pures de ces cellules. Nous avons établi une nouvelle lignée humaine de MC, ROSA^{KIT WT}, dont les propriétés sont similaires à celles des MC primaires, constituant un nouvel outil pour la recherche sur les fonctions des MC humains, et permettant le criblage à haut débit de thérapies anti-allergiques. Les MC sont impliqués dans les mastocytoses, où ils s'accumulent pathologiquement dans divers tissus. Bien que la plupart des cas de mastocytoses systémiques (SM) sont chroniques et indolents, les patients atteints de SM avancée (SM agressive; ASM, et leucémie à mastocytes; MCL) ont un mauvais pronostic, car la plupart des thérapies disponibles ne sont pas curatives. Afin de mieux comprendre la physiopathologie des formes avancées de SM et pour trouver de nouvelles approches pour le traitement, nous avons profité de la disponibilité des cellules ROSA^{KIT WT} pour établir un nouveau sous-clone, la lignée cellulaire ROSA^{KIT D816V}, représentant un équivalent des cellules néoplasiques s'accumulant dans les SM. L'utilisation de cette lignée et de cellules des patients nous a permis d'identifier BRD4 comme une nouvelle cible thérapeutique dans les ASM et les MCL. Nous avons démontré que les MC néoplasiques de patients avec ASM expriment des quantités substantielles de BRD4. Fait intéressant, nous avons aussi démontré que les lignées cellulaires HMC-1 et ROSA^{KIT D816V} expriment aussi BRD4, et que leur prolifération est inhibée par un shRNA BRD4-spécifique. En outre, nous avons montré que le médicament JQ1, inhibiteur de BRD4, induit une inhibition de la croissance et une apoptose dose-dépendante dans les mêmes cellules. De plus, nous avons démontré que JQ1 supprime également la prolifération des MC néoplasiques primaires de patients atteints d'ASM ou de MCL à de faibles concentrations. Enfin, nous avons observé que la midostaurine (PKC412) et l'acide rétinoïque tout-trans (ATRA) coopèrent avec JQ1 pour induire des effets inhibiteurs synergiques sur l'inhibition de la survie des mêmes cellules. En conclusion, nos résultats représentent une avancée sur ce qui était précédemment connu sur l'implication de BRD4 dans les mastocytoses et nous ont permis d'identifier cette protéine comme cible thérapeutique prometteuse dans le traitement des formes avancées de SM.

Title: Identification of BRD4 as a novel therapeutic target in aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL)

Keywords: BRD4, aggressive systemic mastocytosis, mast cell leukemia, therapeutic target

Abstract: Human mast cells (MCs) are hematopoietic stem cell (HSC)-derived, tissue-resident, multifaceted cells involved in a myriad of physiological and pathological processes. Researches on MCs have been hampered for a long time, due to limited access to pure populations of these cells. We have established a new human MC line, ROSA^{KIT WT}, whose properties are similar to those of primary HSC-derived MCs, providing a novel tool for research on human MC functions, and enabling the high-throughput screening of anti-allergic therapies. Among others, MCs are involved in a group of diseases termed mastocytosis, where they accumulate pathologically in various tissues. Although most cases of systemic mastocytosis (SM) are chronic with an indolent course, patients with advanced SM (aggressive SM; ASM, and mast cell leukemia; MCL) have a reduced life expectancy and a poor prognosis, since most of the therapies already available are not curative. In order to better understand the pathophysiology of advanced SM and to find new approaches for treatment, we took advantage of the availability of the ROSA^{KIT WT} cells to establish a new subclone, the ROSA^{KIT D816V} cell line, representing a paradigm of the neoplastic cells accumulating in SM.

Using these malignant cell line and patients' cells, we identified the epigenetic reader bromodomain-containing protein-4 (BRD4) as a novel drug target in ASM and MCL. Indeed, we demonstrated that neoplastic MCs from ASM patients expressed substantial amounts of BRD4. Interestingly, we then demonstrated that HMC-1 and ROSA^{KIT D816V} cell lines express BRD4, and that their proliferation is inhibited by a BRD4-specific shRNA. Moreover, we showed that the BRD4-targeting drug JQ1 induced a dose-dependent growth inhibition and apoptosis in the same cells. In addition, we demonstrated that JQ1 suppressed also the proliferation of primary neoplastic MCs of patients with ASM or MCL at low concentrations. Finally, we reported that midostaurin (PKC412) and all-trans retinoic acid (ATRA) cooperated with JQ1 in producing synergistic inhibitory effects on the survival of HMC-1 and ROSA cells. Together, our data represent a significant advance over what was previously known on the involvement of BRD4 in mastocytosis and identify this epigenetic reader bromodomain-containing protein as a promising drug target in advanced SM.



