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**Functional characterisation of a novel t(3;5)
translocation targeting the Glucocorticoid receptor gene
and a long non-coding RNA in plasmacytoid dendritic
cell acute leukaemia**

Neda Hoghoughi

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THÈSE

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Présentée par

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préparée au sein de **L'équipe 7 « Voies Oncogéniques des Hémopathies Malignes »**

Centre de Recherche INSERM U823 – Université Joseph Fourier
Grenoble 1 – Institut Albert Bonniot
dans **l'École Doctorale Chimie et Science du Vivant**

Caractérisation fonctionnelle d'une nouvelle translocation t(3;5)(q21;q31), ciblant le gène du récepteur aux glucocorticoïdes et un ARN non-codant, dans la leucémie aiguë à cellules plasmocytoïdes dendritiques

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Abstract

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is an incurable malignancy for which disease mechanisms are unknown. Here, we identify the *NR3C1* gene (5q31), encoding the glucocorticoid receptor (GCR), and a long, intergenic, non-coding RNA gene (named here *lincRNA-3q*), respectively, as targets for genetic alteration or transcriptional deregulation in BPDCN. *NR3C1* translocation/deletion was associated to critically short survival in BPDCN and to abnormal activity of GCR, EZH2, and FOXP3 gene regulatory networks. *LincRNA-3q*, was found to encode a nuclear, non-coding RNA that is ectopically activated in BPDCN and high-risk AML. Depletion of *lincRNA-3q* in myeloid cancer cells induced cell cycle arrest, coincident to suppression of E2F1/Rb and leukemia stem cell-specific gene expression signatures. BET bromodomain protein inhibition could selectively suppress *lincRNA-3q* indicating a treatment strategy for counteracting oncogenic activity of this non-coding RNA. Thus, this work defines a new framework for understanding disease pathogenesis and treatment resistance in BPDCN.

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Abbreviations

2HG	2- Hydroxy Glutarate
5-hmC	5-hydroxy methyl- Cytosine
5-mC	5-methyl- Cytosine
AF	Activation Function
ANRIL	Antisense Non-coding RNA in the INK4 Locus
Airn	Antisense insulin-like growth factor 2 receptor RNA
AITL	AngioImmunoblastic T-cell Lymphoma
ALL	Acute Lymphoblastic Leukemia
Allo-SCT	Allogeneic Stem Cell Transplantation
AML	Acute Myeloid Leukemia
APC	Antigen-Presenting Cells
APL	Acute Promyelocytic Leukemia
Ara-C	Cytosine arabinoside
Asx	Additional sex combs
ASXH	amino-terminal ASX Homology
ASXL1	Additional sex combs-Like 1
Auto-SCT	Autologous Stem Cell Transplantation
BD	BromoDomain
BDCA2	Blood Dendritic Cell Antigen 2
BER	Base-Excision Repair
BET	Bromodomain and ExtraTerminal domain
bHLH	basic Helix-Loop-Helix
BM	Bone Marrow
BPDCN	Blastic Plasmacytoid Dendritic Cell Neoplasm

BST2	Bone Marrow Stromal Antigen 2
CBX 7	ChromoBoX 7
cDC	conventional Dendritic Cell
CDP	Common Dendritic Cells Progenitor
CDR	common Deleted Region
CEBPA	CCAAT/Enhancer-Binding Protein Alpha
CGH	Comparative Genomic Hybridization
CHART	Capture Hybridization Analysis of RNA Target
ChIP-seq	Chromatin Immunoprecipitation experiments followed by sequencing
ChIRP	Chromatin Isolation by RNA Purification
CLP	Common Lymphoid Progenitor
CMML	Chronic Myelomonocytic Leukemia
CMPs	Common Myeloid Progenitor
CN-AML	Cytogenetically Normal-Acute Myeloid Leukemia
CNS	Central Nervous System
DBD	DNA-Binding Domain
DC	Dendritic Cell
dChIRP	domain-specific Chromatin Isolation by RNA Purification
DHS	DNaseI-HyperSensitive
DLBCL	Diffuse Large B-Cell Lymphomas
DNMT	DNA MethylTransferase
DT	Diphtheria Toxin
EHMT2	Euchromatic Histone-lysine N-MethylTransferase 2
ENL	Eleven Nineteen Leukemia
ERK	Extracellular signal-Regulated Kinase
ETP	Enhancer of Trithorax and Polycomb
EVI1	Ecotropic Viral integration site 1
EZH2	Enhancer of Zeste Homologue 2
FAIRE-seq	Formaldehyde-Assisted Isolation of Regulatory Elements-sequencing
FAL1	Focally Amplified LncRNA on chromosome 1
FDC	Follicular Dendritic Cell

FISH	F luorescence I n S itu H ybridization
FLT3	F ms- L ike T yrosine kinase 3
FOXP3	F orkhead box P3
GATA1	Erythroid transcription factor or GATA -binding factor 1
GC	G luco C orticoid
GCR	G luco C orticoid R eceptor
GCRFP	G luco C orticoid R eceptor F fusion P rotein
GEP	G ene E xpression P rofile
Gfi-1	G rowth factor i ndependent 1
GM-CSF	G ranulocyte- M acrophage C olony- S timulating F actor
GMP	G ranulocyte/ M acrophage P rogenitor
GRE	G lucocorticoid- R esponse E lement
GSEA	G ene S et E nrichment A nalysis
GSK-3	G lycogen S ynthase K inase 3
HAT	H istone A cetyl T ransferase
HDAC	H istone D e A Cetylase
HDM	H istone D e M ethylase
HMT	H istone M ethyl T ransferase
HOX	H ome O bo X
HSC	H ematopoietic S tem C ell
HSP	H eat S hock P roteins
HSPC	H ematopoietic S tem and P rogenitor C ell
ICL	I nterstrand C ross- L ink
ICR	I mprinting C ontrol R egion
IDH	I socitrate D e H ydrogenase
IFN1	I nter F ero N , type 1
Igf2r	I nsulin-like G rowth F actor 2 R eceptor
IHC	I mmuno H isto C hemistry
IL	I nter L eukin
IRF 8	I nterferon R egulatory F actor 8
IHC	I mmuno H isto C hemistry

ITD	I nternal T andem D uplication
IκB	I nhibitor of NF-κB
JAK2	J ANus K inase 2
JM	J uxta M embrane domain
K	T yrosine- K inase
Kcnq1ot1	Kcnq1ot1 overlapping transcript 1
KI	T yrosine- K inase I nsert
KIT	T yrosine-protein kinase KIT or CD117
KRAS	K irsten R At S arcoma viral oncogene homolog
LBD	L igand- B inding D omain
LC	L angerhans C ell
LILRA4	L eukocyte I mmunoglobulin- L ike R eceptor subfamily A member 4
lincRNA	L ong i ntervening/intergenic n oncoding R NA
lncRNA	L ong n oncoding R NA
LT-HSC	L ong- T erm- H ematopoietic S tem C ell
M-CSFR	M acrophage C olony- S timulating F actor R eceptor
MDP	M acrophage and D endritic-cell P rogenitor
MDS	M yelo D ysplastic S yndrome
MDS/MPN	M yelo D ysplastic S yndrome/ M yelo P roliferative N eoplasm
MEP	M egakaryocyte/ E rythroid P rogenitor
MHC	M ajor H istocompatibility C omplex
MLL	M ixed L ineage L eukemia gene
MNase	M icrococcal N uclease
MPL	M yelo P roliferative L eukemia
MPP	M ulti P otential P rogenitor
ncRNA	n oncoding R NA
NDR	N ucleosome- D epleted R egion
NF-κB	N uclear F actor- κ B
NGS	N ew G eneration S equencing
NK	N atural K iller
NLS	N uclear L ocalization S equences

NPM1	NucleoPhosMin 1 gene
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
O-GlcNAc	O-linked β-D-N-AcetylGlucosamine
OGT	O-GlcNAc Transferase
ORF	Open Reading Frame
p-ALL	pediatric Acute Lymphoblastic Leukemia
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cell
PCAT	Prostate Cancer–Associated ncRNA Transcript
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
pDC	plasmacytoid Dendritic Cell
PHD	Plant HomeoDomain
PTCL	Peripheral T-Cell Lymphoma
PNAcqPCR	Peptide Nucleic Acid clamp real-time Polymerase Chain Reaction
Pol II	Polymerase II
PRC2	Polycomb Repressive Complex 2
Pre-DC	Precursor Dendritic Cell
PTD	Partial Tandem Duplications
PTM	Post-Translational Modification
PTPN1	Protein Tyrosine Phosphatase, Non-receptor type 1
RA	Retinoic Acid
RAP	RNA Antisense Purification
RAR	Retinoic Acid Receptor
RB	RetinoBlastoma
RBD	Ras Binding Domain
RBL1	RetinoBlastoma-Like 1
RBP	RNA Binding Protein
RIP	RNA Immunoprecipitation Assay
RNA-seq	RNA-sequencing
RUNX1	runt-related transcription factor 1

SET	Su(var)3–9/Enhancer of zeste/Trithorax
shRNA	short hairpin RNA
SIGLEC-H	Sialic acid-binding ImmunoGlobulin-like LECTin H
SLO	Secondary Lymphoid Organs
SNP	Single Nucleotide Polymorphism
T-ALL	T cell Acute Lymphoblastic Leukemia
TBP	TATA-Binding Protein
TET	Ten-Eleven Translocation
TET2	Tet methylcytosine dioxygenase 2
TF	Transcription Factor
TKD	Tyrosine Kinase Domain
TLR	Toll-Like Receptor
TM	TransMembrane domain
Topo II	Topoisomerase II
TrxG	Trithorax Group
TSS	Transcription Start Site
WES	Whole-Exome Sequencing
WHO	World-Health Organization
WT1	Wilms-Tumor Protein textbf1
XCI	X Chromosome Inactivation
Xi	X-inactive
Xic	X-inactivation centre
Xist	X-inactive specific transcript
YY1	Yin Yong 1
ZEB2	Zinc fingerE-box-Binding homeobox 2

Chapter 1

Dendritic cells

1.1 Hematopoiesis - General principles

Blood cells are divided into three lineages erythroid (erythrocytes), lymphoid (B and T cells), and myeloid (granulocytes, megakaryocytes, and macrophages). These cells are produced and constantly regenerated in the bone marrow from hematopoietic stem cells (HSCs) through a series of progenitor stages (by a process called hematopoiesis) (Figure 1). The current “deterministic model” for hematopoiesis proposes that Long- Term-Hematopoietic Stem Cells (LT-HSCs) which display a unique ability for life-long self-renewal give rise to Short-Term-Hematopoietic Stem Cells (ST-HSCs), which produce multipotential progenitors (MPPs) that have lost all self-renewal potential but are still able to generate all hematopoietic lineages. From these derive the common lymphoid progenitors (CLPs), which give rise to T, B, NK and certain dendritic cell (DC) cell subsets, and common myeloid progenitors (CMPs)(which give rise to granulocyte/macrophage progenitors (GMPs)), megakaryocyte/erythroid progenitors (MEPs), mast cell and basophil progenitors. This process is tightly controlled by lineage and differentiation stage specific transcription factors that modulate gene expression programs to permanently generate the correct number of specific cell types at a given time and place.

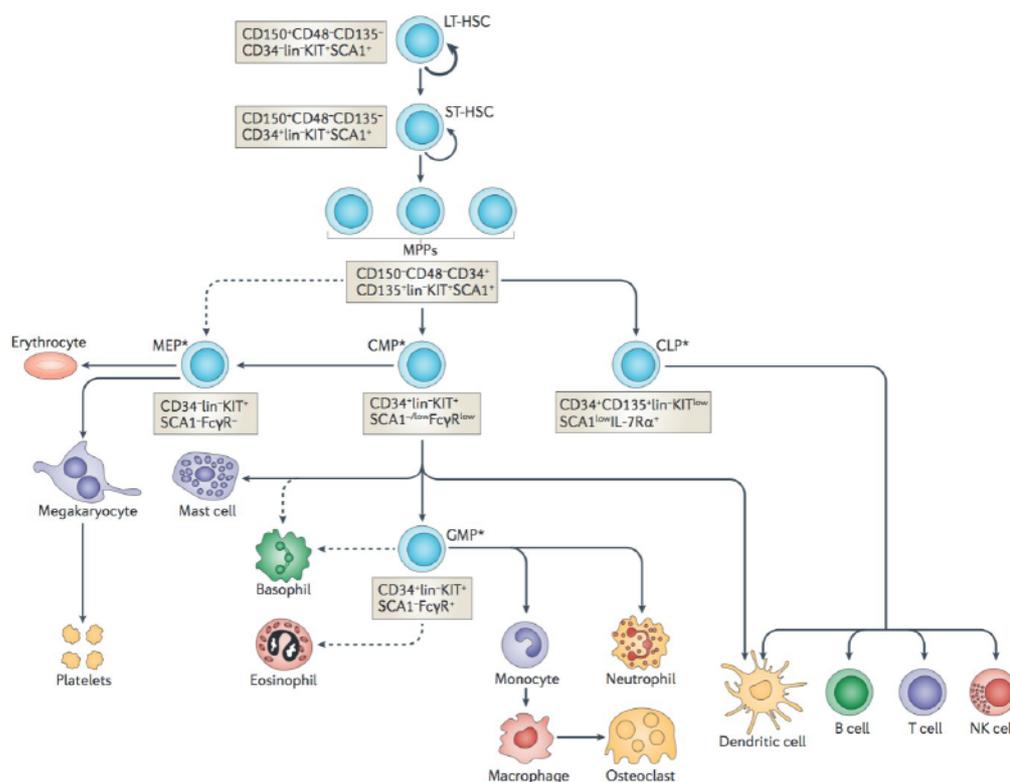


FIGURE 1: Hierarchical model of hematopoiesis in the adult bone marrow. *From (Wang et Wagers, Nat Rev Mol Cell Biol, 2011)*

A comprehensive analysis of the molecular control and the precise interplay of these cell fate decisions are crucial for a full understanding of normal and malignant hematopoiesis (Rieger and Schroeder, 2012). Disruptions of these cell fate decisions underlie hematological disorders including blood cancers.

1.2 Dendritic cell lineage

Dendritic cells (DCs) were first described in 1973 by Ralph M. Steinman and Zanvil A. Cohn (Steinman and Cohn, 1973). DCs are antigen-presenting cells (APCs) of the mammalian immune system and they play a critical role in the regulation of the adaptive immune response. These cells are recognized as the most important APCs with the ability to migrate in lymphatic system and to prime naive T cells in secondary lymphoid organs (SLOs) (Banchereau and Steinman, 1998). Therefore, they constitute a front-line defense against invading pathogens. DCs are located throughout the body and form a sophisticated and complex network that allows them to communicate with

different populations of lymphocytes, thereby forming an interface between the external environment and the adaptive immune system (Belz and Nutt, 2012).

1.3 Dendritic Cell development

In the bone marrow (BM) a fraction of CMP expresses FMS-related tyrosine kinase 3 (FLT3) and differentiates into more-restricted macrophage and DC progenitors (MDPs). MDPs are thought to be the direct precursor to common DC progenitors (CDPs) and give rise to the DC lineages. CDPs produce precursor DCs (pre-DCs) and plasmacytoid DCs (pDCs), which then exit the bone marrow and travel through the blood and migrate toward the secondary lymphoid organs and non-hematopoietic tissues. A limited proportion of DCs may also derive from CLPs in the bone marrow and from early T cell progenitors in the thymus (Belz and Nutt, 2012). The only subsets found in the spleen are lymphoid tissue-resident DCs, which under steady-state conditions arise from pre-DCs. This population is composed of three conventional DC subsets, namely cluster of differentiation 4⁺ (CD4⁺) DCs, CD8 α ⁺ DCs and CD8 α ⁻CD4⁻ double-negative (DN) DCs (Figure 2).

Peripheral lymph nodes contain CD8 α ⁺ and CD8 α ⁻ DC populations but are also populated by two groups of migratory DCs; the dermal DC population which is broadly composed of CD11b⁺ and CD103⁺ DCs, and, migrate through the lymphatic to the lymph node and Langerhans cells that develop in the epidermis and migrate through the basement membrane to the lymph nodes via terminal lymphatic vessels that arise in the dermis. Monocytes arrive at tissues from the blood and in response to inflammation they can develop into monocyte-derived DCs, which adopt many of the characteristics of conventional DCs (Figure 2).

In order to provide their protective function, specialized DCs subsets have evolved and this segregation was initially based on their distinct patterns of cell-surface molecule expression.

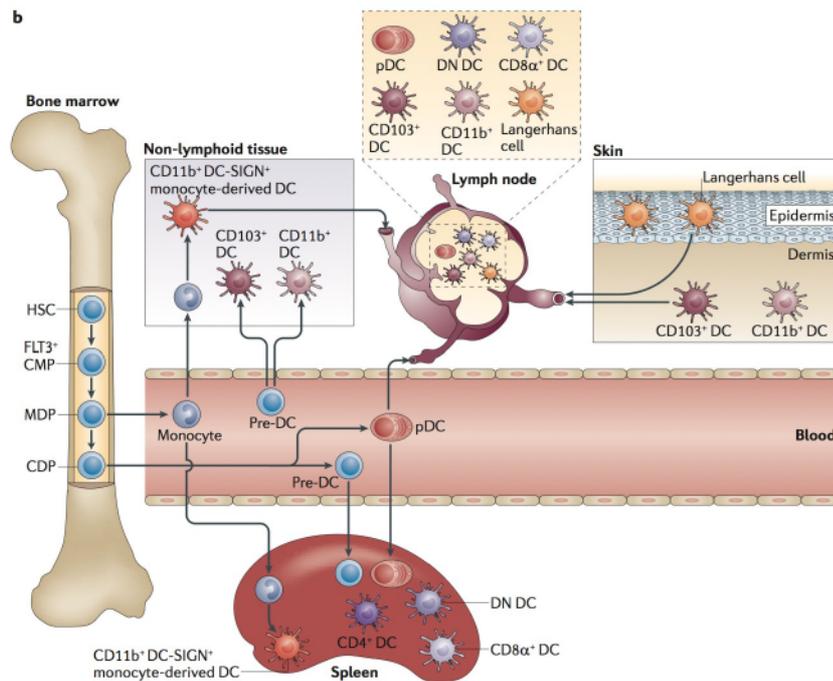


FIGURE 2: Organization of the dendritic cell (DC) network and key surface phenotype markers of different DC subsets, delineated on the basis of their localization in secondary lymphoid tissues. *From (Belz and Nutt, Nat Rev, Immunol, 2012)*

Different subsets of DCs are composed of: plasmacytoid DCs (pDCs), conventional DCs (cDCs), monocyte-derived inflammatory DCs and Langerhans cells (LCs) (Belz and Nutt, 2012).

1.4 Dendritic Cell Subsets

1.4.1 Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells (pDCs) are quiescent cells that are broadly distributed in the body. From their initial identification as “lymphoblasts” by Lennert and Remmele in 1958 (Lennert and Remmele, 1958), pDCs were given a number of different names that suggested a possible cell origin and were exclusively based on their immunophenotype (e.g., plasmacytoid T cells, plasmacytoid monocytes) (Facchetti et al., 1988). Under physiological conditions, pDCs present plasma-like cell morphology, but once activated a radical morphological transformation occurs; the round plasmacytoid cells acquire a

typical dendritic morphology (from the Greek word dendron, for 'tree') that is tightly associated with the specialized effector functions of DC in T cell stimulation (Naik et al., 2005).

pDCs account for <0.1% of peripheral blood mononuclear cells and accumulate in inflammatory sites to contribute to inflammatory and immune response. pDCs are characterized as Lin⁻ MHC-II⁺ CD123 (IL3R)⁺ CD4⁺ CD303(BDCA2)⁺ CD304(BDCA4; Neuropilin-1)⁺ and are known for their capacity for type 1 interferon alpha and beta (IFN α and β) secretion following viral infection by signaling through the nucleic acid-sensing Toll-like receptor 7 (TLR7) and TLR9 (TLR7/9) (Mathan et al., 2013). They are also known for their antigen presentation capacity (Ginhoux et al., 2009, Sasaki et al., 2012), which is of increasing interest for anti-tumoral vaccination strategies.

They express several other characteristic markers, including sialic acid-binding immunoglobulin-like lectin H (SIGLEC-H) and bone marrow stromal antigen 2 (BST2) in mice and blood DC antigen 2 (BDCA2; also known as CLEC4C) and leukocyte immunoglobulin-like receptor, member 4 (LILRA4; also known as ILT7) in humans (Belz and Nutt, 2012). Other surface markers, such as maturation markers appear on mouse pDC as they mature in bone marrow and in the peripheral tissues. Such markers include Ly6C (Vremec et al., 2007), Ly49Q (Toyama-Sorimachi et al., 2005), CD4, and CD8 (O'Keeffe et al., 2002). Human pDCs are usually distinguished from other DC by the lack of expression of CD11c (Dzionic et al., 2000).

More recently, a subset of normal pDCs were shown to express CD56, a minor subpopulation of Lin⁻ DR⁺ CD56⁺ CD123⁺ CD11c⁻ cells were designated as pDC-like cells (pDLCs). These cells constitute 0.03% of peripheral blood mononuclear cells (PBMCs), and express BDCA2, BDCA4, and myeloid antigens, which are frequently expressed by the malignant counterpart of pDC (BPDCN). pDLCs exhibited modest expression of Toll-like receptors and produced less interferon- α after CpG stimulation (Osaki et al., 2013).

1.4.2 Conventional Dendritic Cells

Conventional DCs (cDCs) specialized for antigen processing and presentation. Based on their localization in tissues and their migratory pathways, cDCs can be grouped into two main classes. The first category of conventional DCs is generally referred to as the migratory DCs, which develop from early precursors in the peripheral tissues, where they act as antigen-sampling sentinels, and then they migrate from the peripheral tissues to the lymph nodes. Migratory DCs can be broadly divided into CD11b⁺ DCs (also known as dermal or interstitial DCs) and CD11b⁻ DCs, which have more recently been shown to express CD103 (also known as integrin α E).

Lymphoid tissue-resident DCs are the second major category of conventional DCs that are found in the major lymphoid organs, such as the lymph nodes, spleen and thymus. They can be classified by their expression of the surface markers; CD8 α ⁺ DCs are noted for their major role in priming cytotoxic CD8⁺ T cell responses, CD4⁺ DCs and CD4⁻CD8 α ⁻ DCs can also present major histocompatibility complex (MHC) class I-restricted antigens and more efficiently present MHC class II-associated antigens to CD4⁺ T cells (Belz and Nutt, 2012).

1.4.3 Inflammatory Monocytes

During inflammation, circulating blood monocytes can be rapidly mobilized and can differentiate into cells that possess many prototypical features of DCs, this subset has been described as inflammatory DCs. These cells arise from MDP and are recruited to the site of inflammation where they can present antigens to both CD4⁺ and CD8⁺ T cells (Leon et al., 2007).

Similar to macrophages these cells express a number of markers including macrophage colony-stimulating factor receptor (M-CSFR) (or CD115), which is expressed in monocytes-macrophages and their progenitors, and drives growth and development of this blood cell lineage (Bourette and Rohrschneider, 2000), and respond to growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Segura

et al., 2013). GM-CSF is an important hematopoietic growth factor and immune modulator, which is produced locally by T cells, macrophages, endothelial cells and fibroblasts upon receiving immune stimuli and it can act in a paracrine fashion to recruit circulating neutrophils, monocytes and lymphocytes to enhance their functions in host defense (Shi et al., 2006).

1.4.4 Langerhans Cells

These cells are skin-resident subset of DCs and arise from a bone marrow-derived myelomonocytic precursor that migrates to the lymph nodes to present antigens. They share similarities with macrophages in their gene expression profiles and are developmentally dependent on signaling through the macrophage colony-stimulating factor receptor (Miller et al., 2012). When activated, however, LCs acquire features more in line with cDCs such as their migratory ability and they also share the cDC specific transcription factors (Meredith et al., 2012).

These different populations of DC arise from common lymphoid progenitor (CLP) in the bone marrow and are restricted to macrophage-DC progenitors (MDPs). The MDP is a common precursor that gives rise *in vivo* to monocytes, macrophages, and the two main subsets of DCs: cDC and pDCs (Auffray et al., 2009).

Dendritic cell development is tightly controlled both by cytokines such as FLT3 (Onai et al., 2007), M-CSF and GM-CSF (Fancke et al., 2008) and transcription factors, which will be further described in the section 1.6.

1.5 NK lineage

Natural killer (NK) cells survey host tissues for signs of infection, transformation or stress and, true to their name, kill target cells that have become useless or are detrimental to the host. NK cells, like cytotoxic CD8⁺ T cells (also known as CTLs) are defined based on their cytolytic machinery and the killing of their targets is mediated predominantly via perforin and granzymes. These cells originate from a common lymphoid

progenitor (CLP) and during infection they become activated through antigen-specific receptors and by pro-inflammatory cytokines (such as interleukin-12 (IL-12) and type I interferons (IFNs)), and produce large amounts of IFN γ (Sun and Lanier, 2011). During viral infection, homeostasis is perturbed and mature NK cells become activated, proliferate robustly (Raulet, 2004). cDC and pDCs contribute to NK cell-mediated protection by secreting large amounts of type I IFNs following the triggering of Toll-like receptors (TLRs) and intracellular sensors of viral nucleic acids; indeed, specific pDC ablation leads to decreased NK cell activity (Sun and Lanier, 2011, Swiecki et al., 2010).

1.6 Transcriptional control of Plasmacytoid Dendritic Cell development

The transcription factors (TFs) determining the development of DC subsets have been the subject of intense investigation in recent years. The emerging paradigm is that distinct TFs act at discrete stages to determine or commit distinct lineage choices and cell identities (Figure 3). Some transcription regulators such as Pu.1 (Carotta et al., 2010), Gfi-1 (growth factor independent 1) a transcriptional repressor, and interferon regulatory factor 8 (IRF 8) are broadly required for DC development and they make their specific effects at the level of the DC progenitors. Other transcription factors such as E2-2 for example have more specific effects within the pDC population (Seillet and Belz, 2013).

1.6.1 Ikaros

It has been shown in numerous studies that Ikaros is required at multiple steps in DC development and influences both the earliest DC progenitors and mature DCs to maintain pDC identity (Allman et al., 2006, Onai et al., 2007). Mice expressing low levels of Ikaros lack peripheral pDCs, but no other DC subsets. Loss of pDCs is associated with an inability to produce type I IFN after challenge with Toll-like receptor-7 and -9 ligands. Additionally BM cells of these mice contain a pDC population that appears

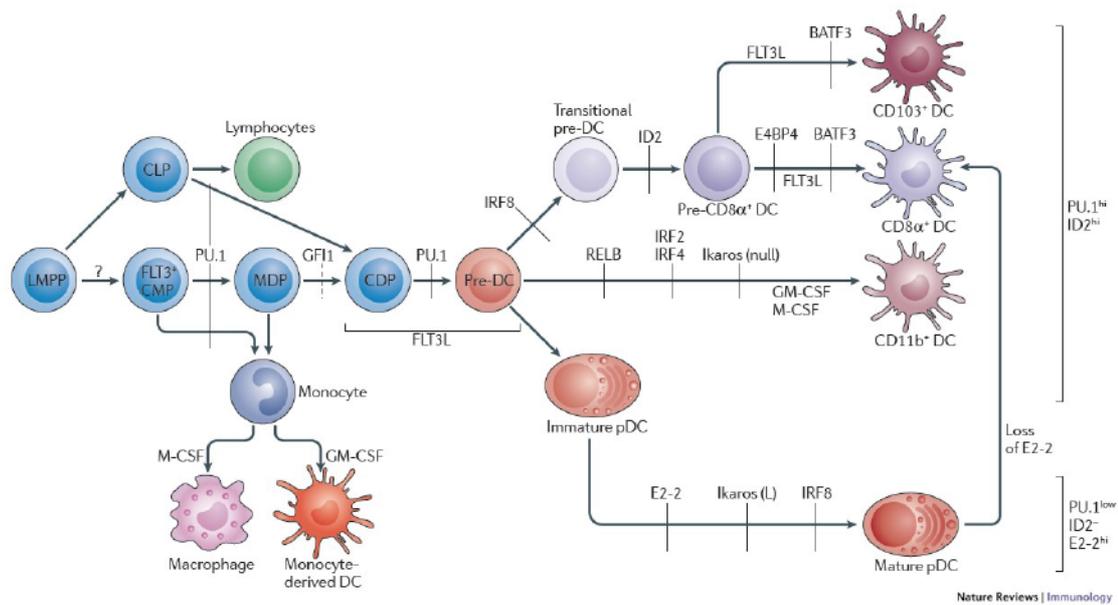


FIGURE 3: Growth factors and transcription factors regulating DC differentiation; the development of both DCs and monocytes depends on high concentrations of PU.1, which regulates the expression of the cytokine receptors FMS-related tyrosine kinase 3 (FLT3), macrophage colony-stimulating factor receptor (M-CSFR) and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR). The plasmacytoid DC (pDC) lineage requires IRF8, a low level of PU.1 and the absence of ID2. The differentiation of pDCs from an immature precursor requires E2-2 and Ikaros, with induced loss of E2-2 converting pDCs into cells that closely resemble CD8 α^+ conventional DCs. CDP, common DC progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; FLT3L, FLT3 ligand; GFI1, growth factor independent 1; LMPP, lymphoid-primed multipotent progenitor; MDP, macrophage and DC progenitor, (From Belz and Nutt, *Nature reviews, Immunology*, 2012)

blocked at the Ly-49Q- stage of differentiation and fails to terminally differentiate in response to Flt-3L (Allman et al., 2006). Ikaros (~50 kDa) encoded by *IKZF1* (*IKAROS family zinc finger 1*) the gene of same name localized to 7p12, is a member of the Kruppel family of zinc finger DNA-binding proteins and is a transcription factor implicated in self-renewal activity in HSC, as well as priming of the B lymphoid transcriptional program (Nichogiannopoulou et al., 1999). Interestingly a number of these genes are also expressed in pDC. The precise downstream targets for Ikaros have not been identified in the pDC lineage.

1.6.2 E2-2

As discussed above, pDCs are a distinct lineage separated from the cDCs, at a late development stage, by differential regulation of discrete gene expression programs by distinct transcription factors, in particular E2-2 (Cisse et al., 2008, Ghosh et al., 2010). E2-2 (~71k Da), encoded by *Tcf4*, located on 18q21 locus, is a member of E-box (or E) proteins that belong to the basic helix-loop-helix (bHLH) transcription factor family. The members of this family bind to regulatory E-box sequences (CATATG/CACCTG) on target genes and activate transcription by forming either heterodimers or homodimers (Kee, 2009). E2-2 is highly expressed in pDC, at lower levels in B cells, and is almost undetectable in other immune cells. pDCs are very sensitive to *E2-2* expression; in *E2-2*^{+/-} mice as in human patients with Pitt-Hopkins syndrome who have *E2-2* haploinsufficiency, pDC numbers and IFN- α secretion are profoundly impaired (Cisse et al., 2008).

E2-2 binds directly to the promoter regions of *Spi-B*, *Irf8*, and *Irf7* genes, which are important factors for pDC development (Cisse et al., 2008, Ghosh et al., 2010). Induced deletion of *E2-2* in mature pDC is associated with the loss of pDCs markers, increased MHC class II expression and T cell priming capacity, acquisition of dendritic morphology, and cDC phenotype accompanied by increased expression of Id2 (an antagonist E protein, which is abundantly expressed in all cDC) and the loss of pDC lineage identity (Ghosh et al., 2010).

1.6.3 SPI-B

SPI-B (~29 kDa), is encoded from *SPI-B* gene located at 19q13 locus, and is an ETS-domain transcription-factor family member and is closely related to PU.1, with 70% sequence identity in their DNA-binding domain (DBD) (Chrast et al., 1995). *SPI-B*-deficient pDCs present defects in TLR7/9-induced type I IFN production, Sasaki et al. demonstrated that pDCs were decreased in BM of *Spi-B*^{-/-} mice whereas peripheral pDCs were increased; this uneven distribution suggested the defective retention of mature non-dividing pDCs in the BM. Therefore *Spi-B* seems to be involved in pDC

function and development in mice (Sasaki et al., 2012). Knock down experiments in CD34⁺ precursor cells demonstrated that the ability of these cells to develop pDCs in both *in vitro* and *in vivo* assays was strongly inhibited (Schotte et al., 2004).

1.6.4 Interferon regulatory factor 8

Interferon regulatory factor 8 (IRF8) (~48 kDa) belongs to the IRF transcription factor family. *IRF8* encoding gene is located at 16q24.1 locus and is particularly highly expressed in common dendritic cell precursor (CDP), pDCs, and CD8a DCs (Becker et al., 2012).

The function of *IRF8* in DC development appears to be conserved across humans and mice. *In vivo* experiments have shown that *IRF8* plays an important role in regulation of proliferation and differentiation of hematopoietic progenitor cells. *IRF8* was recently found to promote CDP development and the initial commitment to the DC lineage by inhibiting granulopoiesis (Becker et al., 2012). pDCs and CD8a DCs help to establish antiviral and antimicrobial responses by producing type I IFN and IL-12, respectively. These functions are impaired in *Irf8*^{-/-} mice, which are more susceptible to viral infection, a phenotype shared by the human patients with *IRF8* mutations (Hambleton et al., 2011).

1.6.5 RUNX2

The Runx family of transcription factors in vertebrates consists of three proteins (Runx1-3). Runx1 (~55 kDa) and Runx3 (~48 kDa) have been extensively studied within the hematopoietic system, where they often act as transcriptional regulators that direct cell fate choices. *Runx1* and *Runx3* are critical regulators of T cell differentiation but *Runx2* has been specifically identified as a master regulator of bone development. It has been recently shown that pDCs specifically express Runx2 (~57 kDa) in an E2-2-dependent manner. *Runx2*, located at 6p21 locus, is required for the expression of several pDC-enriched genes, including the chemokine receptors Ccr2 and Ccr5. Mature pDCs express high levels of Ccr5 at the cell surface, and Ccr5-deficient pDCs are

greatly reduced in the periphery relative to the bone marrow. pDCs in *Runx2*-deficient mice developed normally in the bone marrow but are greatly reduced in the periphery (caused by the retention of mature Ly49Q⁺ pDCs in the bone marrow). These data indicate that *Runx2* facilitates pDC homing to peripheral lymphoid organs in a *Ccr5* dependent manner (Sawai et al., 2013).

1.6.6 L-Myc

MYC genes encode nuclear factors that critically regulate stem cell and metabolic functions, cell proliferation and apoptosis. Their deregulation contributes to the genesis of many human tumors. In mammals, there are four related genes in the family, encoding 4 factors: c-MYC (~67 kDa), N-MYC (~49 kDa), L-MYC (~40 kDa) and S-MYC (~50 kDa). It is very well known now that the enhanced expression of Myc proteins contributes to almost every aspect of tumor cell biology.

The *c-MYC* gene is located at 8q24.21 locus and is the most studied member of this family. Deregulated expression of *c-MYC* occurs in a broad range of human cancers and is often associated with poor prognosis, indicating a key role for this oncogene in tumor progression. *c-MYC* regulates cellular proliferation, metabolism and maintenance of progenitor populations. MYC proteins bind cognate DNA sequences in cooperation with DNA binding partners (MAX) through a conserved basic helix loop helix leucine zipper. At least c-MYC has been shown to directly regulate the transcription of numerous genes but also to control the speed at which transcription proceeds. The third member of myc family of proto-oncogenes is *L-MYC*, which is located at 1p34.2 locus and is also required for normal embryonic development. Forced expression of L-Myc exerts weaker effects than c-MYC for cell growth, apoptosis and transformation (Wasylishen et al., 2011) but is more efficient in reprogramming fibroblasts towards induced pluripotent stem cells (Nakagawa et al., 2010).

Interestingly it has been recently shown that L-Myc is selectively expressed in pDCs and its expression is initiated in common DC progenitor. The knockout experiments in mice have shown that the loss of *L-Myc* in DCs causes a significant decrease

in *in vivo* T-cell priming during infection. It has been shown likewise that some subsets of DCs such as migratory CD103⁺ conventional DCs in the lung and liver are greatly reduced at steady state, when *c-Myc* is replaced by *L-Myc* in immature DCs (Kc et al., 2014).

Chapter 2

Blastic plasmacytoid dendritic cell neoplasm

2.1 Classification and clinical features

Others and we identified leukemic derivatives of pDC in the early 2000s (Chaperot et al., 2001). Following on from this, intense clinical, pathology analysis and biological and functional investigations led to pDC-derived leukemic disorders being classified as a new entity blastic plasmacytoid dendritic cell neoplasm (BPDCN) (formerly called CD4⁺/CD56⁺ hematodermic neoplasm or plasmacytoid dendritic cell leukemia) within AML, in the current WHO classification of hematological malignancies. A characteristic of BPDCN is a clinically aggressive course with a median survival of 12-14 months irrespective of the initial pattern of disease (Fachetti, 2008). BPDCN is a rare disease representing <1% of acute leukemia cases (Jacob et al., 2003), predominantly affects males, with a sex ratio of 3:1, with an average of 67 years old, although some pediatric cases have also been reported (Feuillard et al., 2002). This aggressive disease presents a milder clinical course in children than in adults (Jegalian et al., 2010).

Clinical presentation, consisting at the time of diagnosis of a solitary cutaneous lesion that rapidly disseminates in multiples sites including lymph nodes, bone marrow, blood or central nervous system (CNS) (Jardin et al., 2011). BPDCN cutaneous nodules

are present in 90% of patients (the most common feature; including nodules, plaques, or bruise-like areas) with or without bone marrow, peripheral blood (PB) (60-90%) and lymph nodes (40-50%) involvement. Lymphadenopathy or spleen enlargement or both and cytopenia is frequent (Fachetti, 2008).

Morphologically BPDCN is usually pleomorphic with cell size varying from small to large, with irregular nuclei, fine chromatin and one to several small nucleoli. The cytoplasm is non-granular but displays a heterogeneous structure and cytoplasmic membrane often exhibits pseudopods (Fachetti, 2008, Garnache-Ottou et al., 2007).

As mentioned before, leukemic plasmacytoid dendritic cells have been classified as blastic NK-cell, lymphoma/leukemia or agranular CD4⁺, CD56⁺ and/or hematodermic neoplasm of myeloid precursors (Brody et al., 1995, Fachetti et al., 1990). Chaperot and colleagues were the first to report and recognize that this disease is a malignancy of the pDC lineage (Chaperot et al., 2001). They performed an immunophenotypic study on the leukemic cells of 7 patients (6 men and 1 woman from 8 to 86 years of age, mean = 63 years) and identified that these cells presented expression of the following markers: CD4⁺, CD56⁺, CD3⁻, CD13⁻, CD33⁻, and CD19⁻. They completed these findings with functional studies and demonstrated that, as reported for normal pDCs, in the same culture conditions, leukemic cells became powerful inducers of T-cell proliferation. In addition tumor cells from peripheral blood and lymph nodes of two of these patients produced IFN- α in response to inactivated influenza virus, a distinct feature of pDCs and as such referred to as “natural interferon-producing cells”. On the basis of these observations Chaperot et al. proposed that BPDCN is derived from a plasmacytoid dendritic cell interleukin-3 α (IL-3R α /CD123⁺) precursor. Besides they confirmed that these cells shared also some markers with T (CD4) and NK cells (CD56), suggesting a relation to lymphoid lineage origin of these neoplastic cells (Chaperot et al., 2001, Fachetti, 2008).

Jacob et al. have documented, through an analysis of published cases in literature that an important number of malignant cases showing similar features as explained for BPDCN. They assessed then the expression of more specific pDC-related markers and confirmed that these cells to be HLA-DR⁺, CD123 high, CD116 low, CD45RA⁺,

BDCA2⁺ or BDCA4⁺ (Jacob et al., 2003). In addition, in a study of 45 cases of BPDCN neoplasms and their potential cutaneous mimics, some immunohistochemical markers, such as SPIB, BDCA-4, IRF-8, BCL11A and CD2AP, have been reported as tools for BPDCN diagnosis (Montes-Moreno et al., 2013).

To date the etiology of BPDCN is unclear but its association with myelodysplasia in some cases may suggest a related pathogenesis (Fachetti, 2008). It has been reported that some BPDCN patients presented a prior history of myelodysplastic syndromes (MDS) (Feuillard et al., 2002). MDS is a clonal disorder of hematopoietic stem cells characterized by ineffective hematopoiesis, morphologic dysplasia, peripheral blood cytopenias, and propensity to transformation to acute myeloid leukemia (AML) (Mufti et al., 2003). The genetic features of MDS will be discussed in the following section.

BPDCN, categorized previously as a type of NK-cell lymphoma, is now classified as a rare subgroup of AML and related precursor myeloid neoplasms in the 2008 World Health Organization (WHO) classification 4th edition nomenclature, (Fachetti, 2008) because the origin of the malignancy has been identified as precursor of pDCs (Chaperot et al., 2001).

2.2 Molecular pathogenesis of BPDCN

2.2.1 Chromosomal aberrations

In 2002 Leroux et al. have documented for the first time the cytogenetic features of BPDCN, by using conventional and Fluorescence *in situ* hybridization (FISH) on 18 adults and 3 children with CD4⁺, CD56⁺ DC2 acute leukemia. Clonal, mostly complex chromosome aberrations were found in 14 patients (66%). The aberrant cytogenetic features represented important genomic imbalances, a combination/accumulation of chromosomal anomalies and both lymphoid and myeloid lineage-associated rearrangements (Leroux et al., 2002, Sapienza et al., 2014). This is suggestive of complex multistep tumorigenic mechanisms and is supportive of the hypothesis that BPDCN may arise from an undifferentiated progenitor (Leroux et al., 2002). Leroux et al. showed that two

thirds of patients with BPDCN presented complex karyotypes and documented six major recurrent specific chromosomal abnormalities; 5q21 or 5q34 (72%), 12p13 (64%), 13q13-21 (64%), 6q23-qter (50%), 15q (43%) and loss of chromosome 9 (28%). Recurrent deletions of chromosomal regions such as 4 (4q34), 9 (9p13-p11 and 9q12-q34) and 13 (13q12-q31) have been also observed (Leroux et al., 2002).

In addition to FISH, other approaches have been employed to characterize the genetic landscape of BPDCN. For example, comparative genomic hybridization (CGH) array studies in skin biopsies from 11 cases (5 cases with BPDCN and 6 cases with AML) coupled to Gene expression profiling (GEP), has demonstrated recurrent deletion of regions on chromosome 4 (4q34), chromosome 9 (9p13-p11 and 9q12-q34), and chromosome 13 (13q12-q31) are recurrent in BPDCN (Dijkman et al., 2007) that are distinctive of BPDCN compared to AML with cutaneous lesions. BPDCN was found to show a distinct gene expression profile comprising expression of both myeloid and lymphoid genes compared to AML.

In another study Jardin et al. used array CGH to delineate novel candidate regions and disease-related genes in BPDCN. They studied 9 cases in which the most frequent findings were the loss of chromosome 9 (four cases) or chromosome 13 (five cases). The most frequent partial chromosomal losses affected 5q (four cases), 7p (two cases), 8q (two cases), 9p (two cases), 12p (six cases), 13q (two cases) and 17p (three cases) (Jardin et al., 2009).

Taken together these observations demonstrated that 5q deletion is a frequent phenomenon in BPDCN and it is mostly associated with “lymphoid type” anomalies (up to 70% of cases with abnormal karyotypes) in this disease (Jardin et al., 2009, Leroux et al., 2002, Petrella et al., 2005).

It is well known that deletions involving the long arm of chromosome 5 (chr5), del(5q), are the most common cytogenetic abnormalities in MDS and secondary AML. These chromosomal losses are especially prevalent in AML arising after prodromal MDS or in MDS and AML arising after previous cancer treatment with alkylating agents or radiotherapy (approximately 40% of patients) (Ebert, 2009).

As mentioned briefly before, MDS comprises a heterogeneous spectrum of myeloid malignancies, and the frequent cytogenetic alterations in this disease consists of (-5/5q-, -7/7q-, +8, 20q-, and -Y) (Haase, 2008). Besides 5q- syndrome, which is a hematologic disorder with isolated 5q deletion and blast count of <5% that is associated with specific MDS clinical features and favorable prognosis (Ebert, 2009) a large proportion of del(5q) patients have bone marrow (BM) blasts exceeding 5%, complex cytogenetic, and extremely poor prognosis and are refractory to available treatments (Byrd et al., 2002, Giagounidis et al., 2006, Grimwade et al., 2001).

Chromosome 5q alterations have already been associated to haploinsufficiency mechanisms caused by critical 5q target gene(s) that in cooperation with additional signaling networks drives malignant transformation and clonal evolution in these disorders (Ebert, 2011). It has recently been demonstrated that the deletion of miR-146a in del(5q) MD- S/AML cells is associated with increasing cell survival and proliferation of the propagating cells through the TRAF6/p62/NF- κ B complex (Fang et al., 2014).

Until the present work, no study has addressed identification of 5q driver genes in BPDCN. A significant proportion of BPDCN cases with normal karyotypes are also described but have not undergone detailed molecular investigations either by molecular cytogenetic, sequencing or GEP.

2.2.2 Recurrent gene mutations in BPDCN

2.2.2.1 Tumor suppressor genes

Despite the findings on ontogenetic origin of BPDCN tumors, the genetic causes and oncogenic signaling events involved in the malignant transformation of this disease are still unknown. Preliminary results from molecular studies suggested the presence of abnormalities in genes known to confer a poor prognosis in other hematopoietic malignancies. BPDCN cells display recurrent alterations leading to a combination of deletions of several tumor suppressor genes including: RB1, CDKN1B, CDKN2A, or

TP53 (Jardin et al., 2009), indicating that deletion events altering G1/S regulation are crucial for BPDCN oncogenesis.

TP53

TP53 (*tumor protein 53* or the human *p53* gene) is one of the most commonly mutated tumor suppressor genes in human cancer. Many of these mutations lead to expression of mutant p53 proteins that dominantly interact with normal p53 activity and thereby drive genome instability, abnormal proliferation and cell survival and treatment resistance (Muller and Vousden, 2013). It has been shown that *tp53* inactivation strongly cooperates with oncogenic *Kras* to induce aggressive AML: myeloid progenitor cells expressing oncogenic *Kras* and lacking *p53* become leukemia-initiating cells, resembling cancer stem cells capable of maintaining AML *in vivo*. These results suggested the ability of *tp53* to limit aberrant self-renewal and contribute to its tumor suppressor activity (Zhao et al., 2010). In order to determine the mutational status in BPDCN Jardin et al. performed polymerase chain reaction (PCR) assays followed by direct sequencing on thirteen patients with BPDCN. Five cases displayed *TP53* mutations (38%), leading to changes in its functional domain (Jardin et al., 2011), indicating that *TP53* mutation is a recurrent genetic anomaly in BPDCN.

RB protein families

The *retinoblastoma* (*RB*) gene family is composed of *RB1*, located at chromosome 13q14.2, and two other *RB*-related genes designated as *retinoblastoma-like 1* (*RBL1*) and *retinoblastoma-like 2* (*RBL2*) located respectively at chromosome 20q11.2, a region of special interest because of its association with some myeloid disorders (Claudio et al., 2002) and chromosome 16q12.2 in which deletions or loss of heterozygosity have been reported in several human neoplasms respectively (Di Fiore et al., 2013). These evolutionary conserved genes are structurally related, and play a key role in regulating advancement of the cell division cycle from the G1 to S-phases. They regulate negatively cell cycle entry, by controlling E2F transcription factors and cyclin dependent

kinases. Stimulation of cell cycle entry by growth factor signaling leads to activation of cyclin dependent kinases, resulting in phosphorylation and inactivation of the RB family proteins, and finally E2F activation and DNA synthesis (Figure 4) (Henley and Dick, 2012).

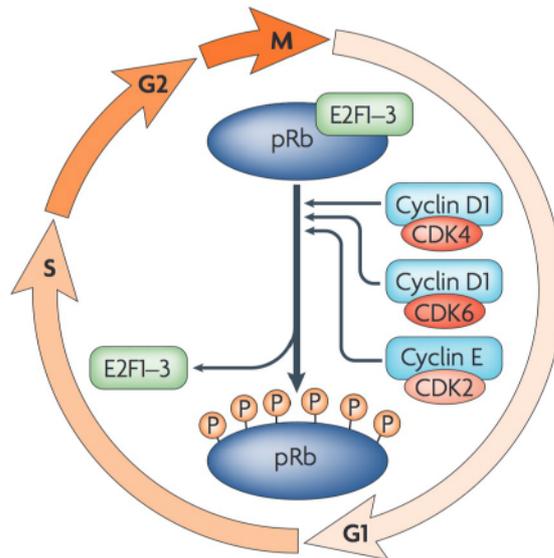


FIGURE 4: The central role of the retinoblastoma protein (pRb) in cell-cycle progression. Entrance into S phase is characterized by phosphorylation of pRb by cyclin D1–cyclin-dependent kinase (CDK)-4, cyclin D1–CDK6 and cyclin E–CDK2 complexes. The phosphorylation of pRb is associated with release of the E2F1–3 transcription factors, which then activate genes that are required for cell-cycle progression. From (Hilary A. Collier, *Nat Rev Mol Cell Biol*, 2007)

It has been demonstrated that mutational inactivation of *RB1* causes the pediatric cancer retinoblastoma, while deregulation of the pathway in which it functions is common in most types of human cancer (Goodrich, 2006). Inactivation of *RB* tumor-suppressor gene has been already reported in lymphoid malignancies (Hangaishi et al., 1996). By using Gene expression profile (GEP) on 11 cases (5 cases with BPDCN and 6 cases with AML), Dijkman et al. demonstrated that there is a diminished expression in oncosuppressor genes such as *RB1* and *LATS2*. *LATS2* is also an important factor for implicated in cell cycle control (Dijkman et al., 2007).

In order to establish correlations with disease outcome and by combining clinico-pathologic findings and genetic data, Lucioni et al. utilized array CGH on 21 cases of BPDCN and they found monosomy of chromosome 13 in 52.4% of samples, they also

detected a common deleted region (CDR) on 13q13.1-q14.3 involving RB1 (Lucioni et al., 2011).

2.2.2.2 Gene mutations affecting cell signaling

FLT3

The *FMS-like tyrosine kinase 3 (FLT3)* gene, located on 13q12, encodes a membrane-bound receptor tyrosine kinase (~140 kDa) that has a crucial role in normal hematopoiesis. It contains five extracellular immunoglobulin-like domains (E), a transmembrane domain (TM), a juxtamembrane domain (JM) and two tyrosine-kinase domains (K) that are linked through the tyrosine-kinase insert (KI) (Figure 5). Cytoplasmic FLT3 undergoes glycosylation, which promotes its localization to the membrane, where it remains as a monomeric, inactive protein on the cell surface until FLT3 ligand (L) binds the receptor and induces receptor dimerization. Its dimerization promotes phosphorylation (P) of the tyrosine-kinase domains, thereby activating the receptor and downstream effectors. FLT3 is normally only expressed in primitive hematopoietic precursors within the bone marrow (Stirewalt and Radich, 2003).

Clinical and experimental evidence both indicate that *FLT3* is a proto-oncogene with the capacity to enhance survival and proliferation of leukemia blast cells (Stirewalt and Radich, 2003). There are two broad categories of activating mutations of *FLT3*. The JM domain of FLT3 often has an autoinhibitory function, therefore mutations disrupting this domain and causing the deformation of the secondary structure of FLT3, result in constitutive activation of the tyrosine kinase domain of this receptor (the so-called *FLT3-ITD* “*internal tandem duplication mutations*”). Other major *FLT3*-activating mutation consists of point mutations within the tyrosine kinase domain (TKD), particularly on the activation loop at aspartate 835 (D835) (Grunwald and Levis, 2013) (Figure 5). The constitutive activation of FLT3 promotes ligand-independent cell proliferation and blocks myeloid differentiation of early hematopoietic cells (Stirewalt and Radich, 2003). Mutations in the *FMS-like tyrosine kinase* gene characterize more than 30 % of AML cases. *FLT3 internal tandem duplication (ITD)* mutations, accounting for

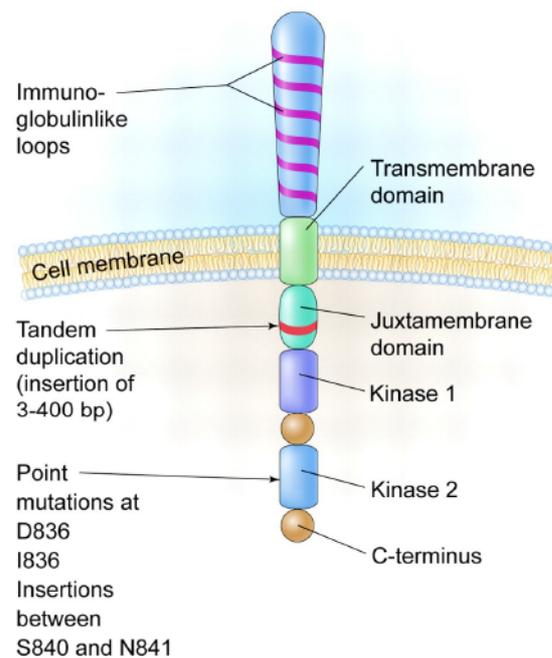


FIGURE 5: A schematic diagram of the FLT3 receptor tyrosine kinase showing the location of the internal tandem duplication of genes within the juxtamembrane domain and point mutations and gene insertions in the second kinase domain. Illustration by Kenneth Probst. From (Mark R. Litzow, *Blood*, 2005)

approximately 23 % of AML cases, are associated with a particularly poor prognosis (Grunwald and Levis, 2013).

Pagano et al. have done molecular analysis on 14 BPDCN patients and found that three (21%) of these patients had *FLT3-ITD* mutations (Pagano et al., 2013). Gene-expression profiling on 8 BPDCN patient biopsies also demonstrated that there is a high expression of FLT3 in some of these samples, and it was paradoxically associated with monoallelic deletion of the locus containing this proto-oncogene (Dijkman et al., 2007).

BRAF

The RAF-MEK-ERK signal transduction pathway is a conserved RAS-activated protein kinase cascade that regulates cell growth, proliferation, and differentiation in response to growth factors, cytokines, and hormones with RAF as the first effector identified downstream of RAS (Malumbres and Barbacid, 2003).

RAF activation initiates by RAS-GTP association with the N-terminal RAS binding domain (RBD), which is associated by concomitant conformational changes and recruitment to the cell membrane, promoting changes in RAF phosphorylation, stimulating its serine/threonine kinase activity. This results in the triggering sequential phosphorylation and activation of MEK and extracellular signal-regulated kinase (ERK) (Wan et al., 2004). Constitutive activation of the RAS-ERK signaling pathway is common to numerous cancers. Almost 90% of *BRAF* mutations result in a Val⁶⁰⁰Glu (V600E) amino acid substitution leading to constitutive kinase activation (Hoeflich et al., 2006).

Recently Go et al. examined *BRAF* mutation by using Sanger sequencing and peptide nucleic acid clamp real-time polymerase chain reaction (PNAcqPCR) in a series of 129 cases of histiocytic, dendritic cell and other related lesions. PNAcqPCR is a sensitive method for detection of mutations in the presence of a large excess of wild-type DNA. PNA is a synthetic DNA analogue, in which the ribose/phosphate backbone of the DNA has been replaced by N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA binds strongly to complementary DNA by Watson-Crick base pairing, whereas one single mismatch will severely destabilize the complex.

This analysis detected *BRAF* V600E mutations, and Histiocytic sarcoma exhibited the highest rate of *BRAF*^{V600E} (62.5%, five of eight), followed by Langerhans cell tumors (25%, seven of 28), follicular dendritic cell sarcoma (18.5%, five of 27) and giant cell tumor (6.7%, two of 30) (Go et al., 2014).

2.2.2.3 Transcription factors

Ikaros

By using a whole-exome sequencing (WES), Meneses et al. provide the first mutational profiling of BPDCN. They identified recurring mutations for a group of newly identified genes, such as *IKZF3* (Ikaros family of zinc-finger protein hematopoietic-specific transcription factors involved in the regulation of lymphocyte development) and *ZEB2*

(Zinc finger E-box-binding homeobox 2, is a transcription factor implicated in development), with prevalence range of 12-16% in BPDCN. It is of note that *Ikaros* mutations are also described in B-ALL (Kastner et al., 2013, Menezes et al., 2014).

2.2.2.4 Epigenetic regulators

Beside recurrent mutations that confer a growth advantage by activating downstream effectors of various signaling pathways and mutations that impact the expression of key transcriptional targets in myelopoiesis, emerging genetic data suggest that there are additional classes of mutations that commonly occur in patients with myeloid malignancies. The most prominent examples are from recent genome-wide and candidate-gene studies that have identified somatic alterations in genes that encode proteins regulating DNA methylation and post-translational histone modifications. These data suggest that somatic alterations in epigenetic regulators are a common genetic event in cancer including myeloid malignancies and contribute to hematopoietic transformation (Shih et al., 2012).

In the following section I briefly introduce epigenetics deregulation, which is an important emerging hallmark of cancer, and will discuss specifically the mutations affecting epigenetic factors that have been demonstrated to be associated to BPDCN and similar hematological malignancies.

Epigenetics: an emerging hallmark of cancer

As normal cells evolve progressively to a neoplastic state, they acquire a succession of capabilities that help them to become tumorigenic and ultimately malignant. Tumors are complex tissues composed of multiple distinct cell types that interact with one another, and tumor microenvironment contributes actively to their development. As it proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2011), the hallmarks of cancer consist of the following biological capabilities; sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, genome instability and mutation, tumor-promoting inflammation, inducing angiogenesis, activating

invasion and metastasis. They have also proposed two emerging hallmarks based on recent research, comprising; the deregulation of cellular energetics and the capability of avoiding immune destruction in their last model (Figure 6).

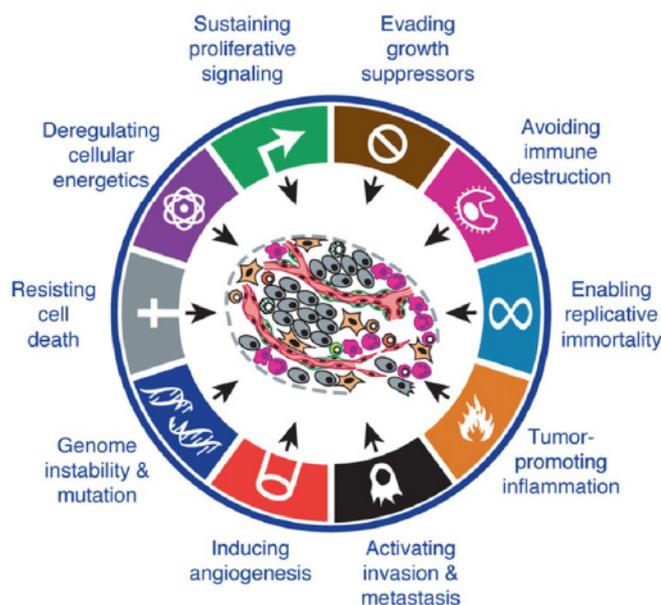


FIGURE 6: The hallmarks of cancer. *From (Hanahan and Weinberg, Cell, 2011)*

In the past decade a remarkable acceleration has been made in the validation of the concept as well as genetic anomalies that result in tumor formation and progression, cancer is an epigenetic disease (Baylin and Jones, 2011). Following the discoveries of DNA methylation abnormalities, learning about chromatin covalent modifications, their organization and relevance to gene expression, brought us to the emerging view of what is now called “the cancer epigenome”. This comprises alterations in epigenetic information as opposed to DNA sequence changes. By definition epigenetic alterations are heritable and somatically reversible making them ideal targets for treatment intervention in cancer.

General overview on epigenetics

The genetic code resides within a negatively charged DNA polymer. The binding of the DNA fiber around basic histone proteins results in neutralizing the negative charges

and allows the DNA to be folded into chromosomes and compacted up to 10,000 times within the nucleus. Wrapping DNA around histone core, which is composed of two copies of each of the histone proteins H2A, H2B, H3 and H4, forms the most basic unit of eukaryotic chromatin: the nucleosome (Dawson and Kouzarides, 2012) (Figure 7).

Histones are not merely DNA-packaging proteins, but molecular structures that participate in the regulation of gene expression. They store epigenetic information through post-translational modifications on their amino-terminal histone “tails”, as lysine acetylation, arginine and lysine methylation, and serine phosphorylation. These modifications affect gene transcription and DNA repair (Jiang and Pugh, 2009).

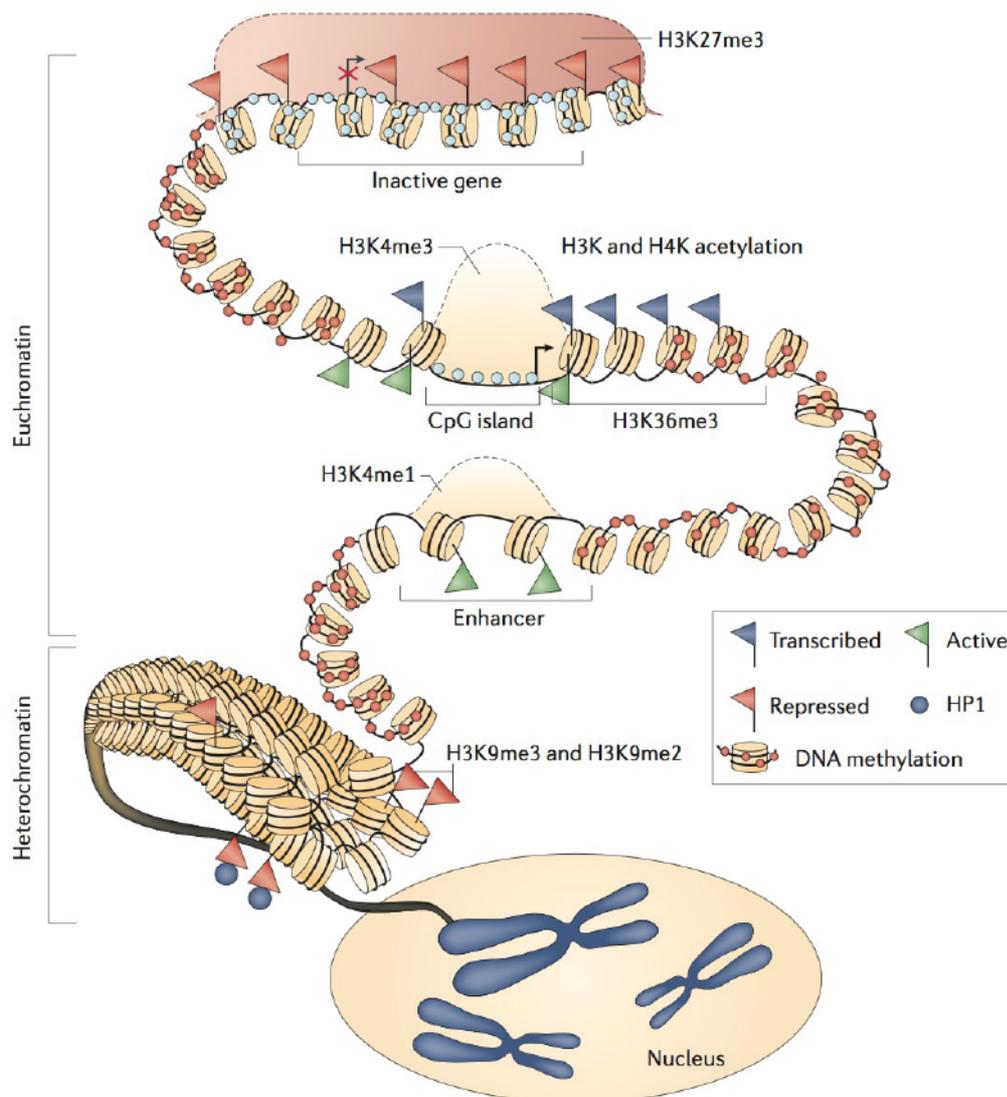


FIGURE 7: Overall structure of the epigenome in human cells. *From (Baylin and Jones, Nat Rev Cancer, 2011)*

Today we know that nucleosome positions are dynamic and change during cell replication and gene expression (Kelly et al., 2010). Active gene promoters are usually characterized by CpG-rich without DNA methylation and having nucleosome-depleted regions (NDRs) upstream of their transcription start sites (TSSs). The flanking nucleosomes of these NDRs are marked by the histone modifications such as H3 trimethylated on lysine 4 (H3K4me3), H3 acetylated on lysine 27 (H3K27ac), extensive lysine acetylation and harbor the histone variant H2A.Z and H3.3, which may destabilize nucleosomes to facilitate transcriptional initiation. Gene bodies of active genes also show enrichment of specific covalent marks, such as H3K36me3, which may facilitate transcriptional elongation (Guenther et al., 2007). By contrast, DNA methylation in promoter regions, in general enhances epigenetic gene silencing, with nucleosomes positioned over the TSS harboring repressive histone modifications, such as H3K9me2, H3K9me3, H4K20me3 and H3K27me3 marks and preventing gene transcription (Baylin and Jones, 2011) (Figure 7). Highly compacted, transcriptionally silent chromatin is known as heterochromatin, whereas more accessible chromatin is known as euchromatin. Heterochromatin can be divided into i) constitutive, which designates genomic regions in the nucleus of a eukaryotic cell that have the opportunity to adopt open or compact conformations within temporal and spatial contexts (Trojer and Reinberg, 2007) or ii) facultative that mainly comprises repetitive genetic elements, such as telomeres and centromeres, that localize to the nuclear periphery (Oberdoerffer and Sinclair, 2007).

Epigenetic readers, writers and erasers

As mentioned above, epigenetic modifications of DNA and histones, and/or alterations in chromatin-remodeling processes, determine active and repressive chromatin states of genes and thus operate as switches either to turn gene expression “on” or “off”. These alterations can be as important for tumorigenesis as the altered genome itself. Each of these epigenetic pathways involves enzymes that transfer modifications (“writers”), which establish a mark on either DNA or the histone tail. These modifications can be removed or modified by enzymes reverting modifications (“editors”), and the third class

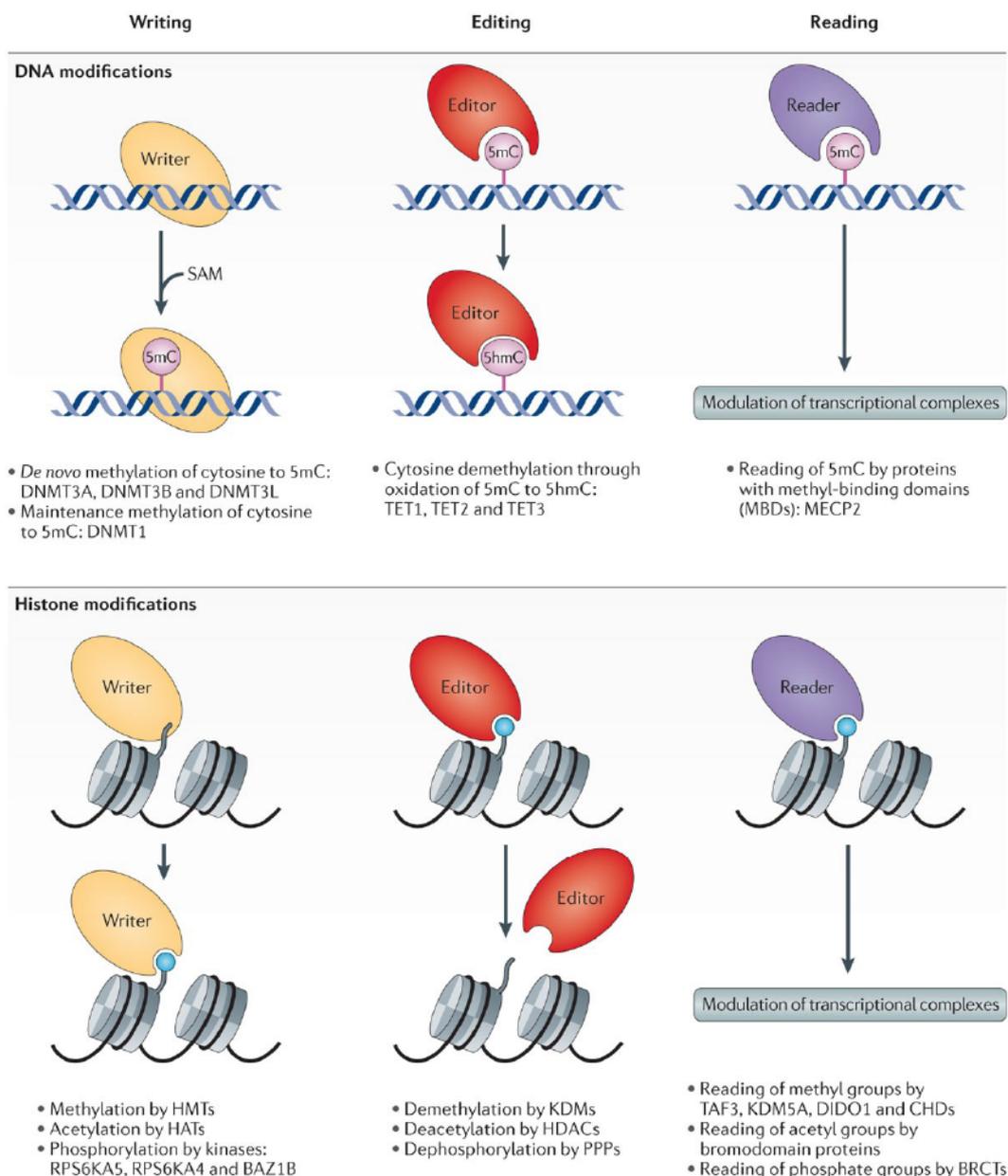
of enzymes is composed of “readers” of epigenetic marks, which mediate the interaction of these marks with a protein complex and thus influence transcription (“readers”) (Figure 8). Readers recognize modifications on histone tails and subsequently translate the information into distinct transcriptional profiles through alterations of chromatin states. They determine whether a chromosomal region is accessible for the binding of transcription factors or other regulatory molecules. The modification patterns are established by histone acetyltransferases (HATs) or histone methyltransferases (HMTs) and can be removed by histone deacetylases (HDACs) or histone demethylases (HDMs). These enzymes are usually specific to particular amino acid motifs on N-terminal histone tails, core histones and histone variants (Plass et al., 2013).

As mentioned earlier, it is well known now that mutations in genes encoding epigenetic regulators are a common genetic event in myeloid malignancies and contribute to hematopoietic transformation. These mutations target all major classes of epigenetic regulator including *tet methylcytosine dioxygenase 2 (TET2)*, *isocitrate dehydrogenase 1 (IDH1)*, *IDH2*, *additional sex combs-like 1 (ASXL1)*, *enhancer of zeste homologue 2 (EZH2)* and *DNA methyltransferase 3A (DNMT3A)*, which have recently been shown to have biological, clinical and potential therapeutic relevance in myeloid malignancies (Shih et al., 2012). Some of these somatic alterations of epigenetic regulators genes will be specifically discussed in the following section because of their relevance to BPDCN pathogenesis.

2.2.2.5 Mutations affecting *DNMT3A*, *TET2* and *IDH2*

DNMT3A

DNA methylation is one of the best-characterized epigenetic modifications. It is an essential process for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, suppression of repetitive elements and carcinogenesis (Jones and Takai, 2001). DNA methylation consists of addition a methyl group (CH₃) on carbon 5 (C5) of cytosine (5-methylCytosine) on DNA fiber. In mammals, DNA methylation occurs on a cytosine followed by a guanine



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FIGURE 8: Enzymes involved in DNA and histone modification pathways. The top panel depicts DNA modifications, such as DNA methylation and demethylation, and the enzymes involved; the bottom panel shows histone modifications and the enzymes involved. Examples for each class of enzyme are given. 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; BAZ1B, tyrosine protein kinase BAZ1B; BRCT, BRCT domain-containing protein; CHD, chromodomain helicase DNA-binding protein; DIDO1, death-inducer obliterator 1; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; KDM, lysine-specific histone demethylase; MECP2, methyl-CpG-binding protein2; PPP, serine/threonine; RPS6K, ribosomal protein S6 kinase; SAM, S-adenosyl-l-methionine; TAF3, transcription initiation factor TFIID subunit 3; TET, TET 5mC hydroxylase. *From (Plaas C. et al., Nat Rev Genet, 2013)*

(CpG) through the DNA sequence and about 70-80% of CpGs are methylated. CpGs are not found frequently in human genome and they are mostly present on gene promoter regions in clusters that are called CpG islands (Weber et al., 2007). Levels of DNA methylation correlates with chromatin accessibility, and acquired abnormal methylation, which can be due to mutation in genes controlling the DNA methylation factors (Figure 9) is a common event in divers diseases including cancer. This results in transcriptional disorders and can be inherited by daughter cells (Jones and Takai, 2001). It is well known that 5mC is associated with a transcriptionally repressed chromatin state, and DNA methylation at specific genomic loci, including lineage-specific genes, can help to shape the cellular identity during development (Kohli and Zhang, 2013).

The enzymes responsible for this modification, DNMTs, are grouped in two categories: de novo and maintenance DNMTs. Initially the DNA methylation patterns are established by the de novo DNA methyltransferases: DNMT3A, (~102 kDa), and DNMT3B, (~96 kDa), during embryonic development (Law and Jacobsen, 2010). These methyl marks are then maintained during cell divisions through the action of the maintenance methyltransferase, DNMT1, (~183 kDa), which preferentially methylates the unmethylated strand of hemimethylated DNA during DNA replication. These mechanisms are crucial for development, since mice deficient in *DNMT3B* or *DNMT1*, located respectively on 20q11 and 19p13 loci, are embryonically lethal and *DNMT3A*-null mice die at 4 weeks of age (Wu and Zhang, 2010).

Ley and colleagues performed whole-genome sequencing on AML samples with normal cytogenetic and identified for the first time somatic mutation of *DNMT3A*. *DNMT3A* gene, located on 2p23 locus, was found mutated in 22%, (62 of 281) of AML cases, and these mutated cases were associated with a poor outcome (Ley et al., 2010). In another study, the direct sequencing demonstrated also that *DNMT3A* was mutated in 87 (17.8%) of 489 patients with AML (Thol et al., 2011).

Exome sequencing in M5 subtype of AML demonstrated that (20.5%) 23 of 112 cases presented *DNMT3A* mutations and these mutants showed reduced enzymatic activity or aberrant affinity to histone H3 *in vitro*. The majority of somatic *DNMT3A* mutations result either in premature truncation of the protein product (nonsense or

frameshift mutations), or occur at a single amino acid, R882 (60% of mutated cases, 37 of 62 mutations) and has been shown to decrease catalytic activity and DNA binding affinity (Gowher et al., 2006). These findings suggest a contribution of aberrant DNA methyltransferase activity to the pathogenesis of acute monocytic leukemia (Yan et al., 2011).

DNMT3A mutations have also been observed in BPDCN patients, Menez et al. have assessed whole-exome sequencing (WES) on 3 BPDCN cases and on the basis of these data, they designed a resequencing approach to identify mutations in 38 selected genes in 25 BPDCN patients. They observed that half of the tumors had mutations affecting either the DNA methylation or chromatin-remodeling pathways, with mutated *DNMT3A*, observed in BPDCN cases. *DNMT3A* mutations were then correlated with a poor prognosis in these patients (Menezes et al., 2014).

TET2

TET2 coding gene is located on 4q24 locus and codes for TET2 protein (~220 kDa), one of the three proteins of the TET (Ten-Eleven Translocation) family, which are evolutionarily conserved dioxygenases that catalyze the conversion of 5-methyl-cytosine (5-mC) to 5-hydroxymethyl-cytosine (5-hmC) (Figure 9) and promote DNA demethylation. In addition to their hydroxylase activity, TET proteins recruit the O-linked β -D-N-acetylglucosamine (O-GlcNAc) transferase (OGT) enzyme to chromatin, which promotes post-transcriptional modifications of histones and facilitates gene expression (Tahiliani et al., 2009). Pathways for active DNA demethylation include the oxidation of 5mC by TET 5mC hydroxylases.

Alternative mechanisms to oxidative demethylation of cytosines are based on deamination and subsequent base-excision repair (BER) or nucleotide-excision repair. These demethylation pathways are replication-independent mechanisms allowing rapid conversions from methylated to unmethylated states.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from benign proliferating cells to an invasive cancer. Three abnormalities are caused by DNA hypomethylation in cancer cells: the generation of chromosomal instability, the reactivation of transposable elements and loss of imprinting. Hypomethylation of DNA can also favor mitotic recombination, leading itself to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused aneuploidy. Activation of intragenomic endoparasitic DNA, such as L1 (long interspersed nuclear elements), and Alu (recombinogenic sequence) repeats is another consequence of undermethylated DNA. These hypomethylated transposons can be translocated to other genomic regions and cause further disruption in the genome (Esteller, 2008).

TET2 gene mutations are recurrent in myeloid and lymphoid malignancies and accumulate with age. It has been recently demonstrated that alterations of *TET2* may represent an important and early event in the pathogenesis of various myeloid malignancies including MDS, chronic myelomonocytic leukemia (CMML), or de novo/secondary AML (Mullighan, 2009).

It has been also shown that acquired *TET2* mutations could be detected at a low level in CD3⁺ cells of MDS patients, indicative therefore of a very early event during hematopoiesis (Smith et al., 2010). Missense, frameshift and nonsense mutations in *TET2* also occur in 7.6% of AML samples. Mutation rates in myeloproliferative neoplasms are higher (>20%), but these rates may be biased, as the number of tested samples is smaller (Plass et al., 2013).

In another study direct sequencing on a cohort of 190 peripheral T-cell lymphomas (PTCL) demonstrated that *TET2* mutations were presented in 40 of 86 (47%) cases of angioimmunoblastic T-cell lymphoma (AITL) and in 22 of 58 (38%) cases of peripheral T-cell lymphoma, but were absent in all other PTCL entities, with the exception of 2 of 10 cases of enteropathy-associated T-cell lymphoma (Lemonnier et al., 2012).

Interestingly *TET2* mutations have been described in BPDCN. Briefly, by direct sequencing on thirteen patients with BPDCN, *TET2* mutations were detected in (7/13)

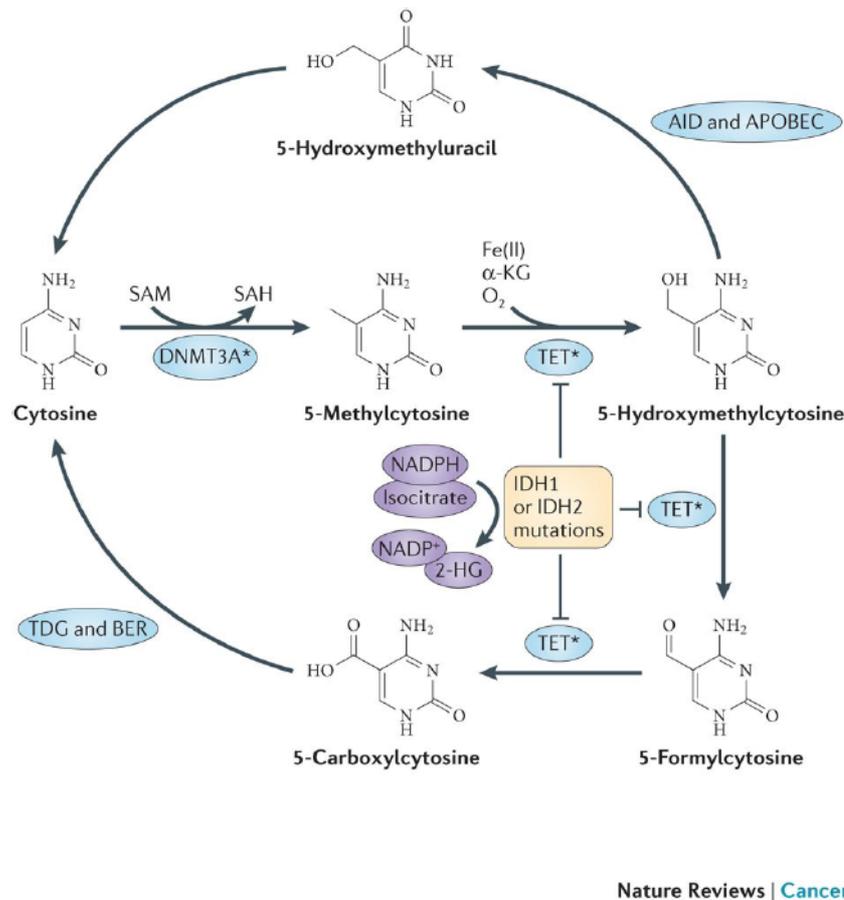
cases (53%) (Jardin et al., 2011). Menez et al. by using next generation sequencing (NGS) analyzed 42 BPDCN patients and found that 36% of these patients presented *TET1/2* mutations.

Also in a recent study and by using NGS on a panel of recurrently mutated genes in AML, it has been suggested that an appreciable subset of BPDCN cases have *TET2* mutations (Alayed et al., 2013). In this study NGS were performed on 5 of these patients and *TET2* mutations were found in 4 cases (80%). This raises the possibility that perturbations in DNA methylating modifying enzymes, here *TET2* mutations, play a role in the pathogenesis of BPDCN (Alayed et al., 2013).

The major functional consequence of *TET2* mutation is a hypermethylation phenotype thus indicating that *TET2* mutated cancers should respond to hypomethylating agents (Shih et al., 2012).

IDH2

The homodimeric enzymes, Isocitrate dehydrogenases 1 and 2 (IDHs 1 and 2), IDH1 (~235 kDa) and IDH2 (~51 kDa), both catalyze the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG) and reduce NADP^+ to NADPH, but they have different subcellular locations: IDH1 in the cytoplasm and IDH2 in mitochondria. Their enzymatic activity is then followed by a series of additional enzymes, which further process 2-ketoglutarate to produce energy in cells. *IDH1* and *IDH2* genes are located on 2q33.3 and 15q26.1 loci respectively. *IDH1* mutations were discovered in patients with acute myeloid leukemia (AML) through whole genome sequencing (Mardis et al., 2009, Ward et al., 2010). Sanger sequencing and Sequenom MassAR- RAY platform (Sequenom) have demonstrated that *IDH2* mutations were present in angioimmunoblastic lymphoma cases (Cairns et al., 2012). Mutations are heterozygous and occur at the arginine residue of the enzyme's active site and cause both loss of normal enzyme function and gain of function (McKenney and Levine, 2013). *IDH* mutations induce a neomorphic function to produce the oncometabolite 2-hydroxyglutarate (2HG), which can



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FIGURE 9: Associated mutations in the DNA methylation and demethylation pathway. The process of DNA methylation and the enzymes involved are shown. Enzymes affected by somatic mutations in myeloid leukaemia, are marked with an asterisk. AID, activation-induced cytidine deaminase; BER, base-excision repair; DNMT3A, DNA methyltransferase 3A; IDH, isocitrate dehydrogenase; NADP⁺, nicotinamide adenine dinucleotide phosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TDG, thymine DNA glycosylase, 5fC (5-formylcytosine), 5caC (5-carboxylcytosine). From (Shih et al., Nat Rev Cancer, 2012)

alter the epigenetic landscape. *IDH* mutations are most common in patients with cytogenetically normal AML (CN-AML), and the prevalence of *IDH1* and *IDH2* missense mutations among patients with AML is between 5% and 20% (McKenney and Levine, 2013). By using WES, Menez et al. analyzed a total of 25 BPDCN patients and reported (1/25) 4% and (3 of 25) 12% of these cases were mutated for *IDH1* and *IDH2* genes respectively (Menezes et al., 2014).

2.2.2.6 Mutations affecting histone-modulating factors

MLL

Mixed lineage leukemia (MLL) gene is located at chromosome 11, band q23 and encodes a histone methyltransferase that directly binds DNA and positively regulates gene transcription, and particularly regulates the expression of *homeobox (HOX)* genes. MLL is a member of Trithorax group (TrxG) of proteins and is known because of its involvement in leukemia (Schuettengruber et al., 2011).

MLL (~180 kDa) is a large multi-domain protein, ubiquitously expressed in hematopoietic cells including stem and progenitor populations. Rearrangements of *MLL*, the human homolog of the *Drosophila* gene *trithorax*, are associated with aggressive lymphoid and myeloid acute leukemias in both children and adults. MLL mutations are found as a fusion protein that has no H3K4 methylation function (Krivtsov and Armstrong, 2007). *MLL* fusions are highly efficient oncoproteins that transform hematopoietic progenitors and cause aggressive leukemia (Slany, 2009). Approximately 10% of human acute leukemias harbor *MLL* translocations and *MLL* is also affected by abnormalities such as partial tandem duplications (PTDs) of exons encoding the N terminus of MLL in about 10% of normal cytogenetic AML cases (Muntean and Hess, 2012).

Depending on the fusion partner, they seem to either affect chromatin-associated processes or, more frequently, specifically stimulate transcriptional elongation (Maethner et al., 2013). “Eleven Nineteen Leukemia” protein (ENL) originally discovered as an MLL fusion partner in the recurrent translocation t(11;19) (Maethner et al., 2013). ENL belongs to a protein complex called EAP as “elongation assisting proteins”. It has been demonstrated that EAP has also the capacity to accommodate MLL fusion proteins. Therefore the constitutive recruitment of EAP to MLL target loci is responsible for persistent target transcription through stimulation of transcriptional elongation. This mechanism resists differentiation stimuli and therefore causes a maturation arrest (Mueller et al., 2009). The *MLL* rearrangements have been also reported in a BPDCN case with *MLL-ENL* fusion gene, suggesting its implication in the development of this disease (Toya et al., 2012). Two BPDCN cases with 11q23 abnormalities were also

reported in another study on 5 cases of DC malignancy, which carried t(9;11)(p21; q23) and 11q23 deletion (Bueno et al., 2004).

Mutations affecting the Polycomb repressive complex PRC2

Cellular memory is provided by two counteracting groups of chromatin proteins termed Trithorax group (TrxG) and Polycomb group (PcG) proteins. The antagonistic activities of the PcG and the trithorax families of proteins culminate in the maintenance, throughout development and adulthood, of the appropriate patterns of homeotic gene expression in a spatially defined manner. TrxG proteins activate transcription and PcG complex is implicated in gene repression (Schuettengruber et al., 2011). In mammals, two main PcG complexes exist, Polycomb repressive complex 1 (PRC1) and 2 (PRC2). The PRC2 complex is responsible for the methylation (di- and tri-) of Lys 27 of histone H3 (H3K27me_{2/3}) through its enzymatic subunits enhancer of zeste homologue, EZH1 and EZH2, whereas the PRC1 complex monoubiquitylates Lys 119 of histone H2A (H2AK119ub) via the ubiquitin ligases RING1A and RING1B. It has been suggested that PRC1 functions downstream of PRC2, and both are often required to maintain gene repression (Margueron and Reinberg, 2011).

The core PRC2 complex, which is conserved from *Drosophila* to mammals, comprises four components: EZH1/2, SUZ12, EED and RbAp46/48 (also known as RBBP7/4) (Figure 10). It has been demonstrated that besides the EZH2 methyltransferase activity through its SET (Su(var)3-9/enhancer of zeste/trithorax) domain, SUZ12 is also important for H3-K27 methylation and gene silencing (Cao and Zhang, 2004) and HMTase activity requires a minimum of three components-EZH2, EED, and SUZ12-while AEBP2 is required for optimal enzymatic activity (Cao and Zhang, 2004). The PRC2 component EED, can bind H3K27me₃, and PRC2 enzymatic activity is stimulated by the presence of H3K27me₃, thus generating a positive-feedback loop. H3K27me₂ and H3K27me₃ marks are associated with facultative heterochromatin: a subdivision of heterochromatin that is regulated in a developmental-specific manner. Acetylated H3K27 is thought to be antagonistic to PcG-mediated silencing and is enriched in the absence of PRC2.

A model predicting the recruitment of PRC2 to chromatin has been proposed: first the interaction of JARID2 and AEBP2 with DNA, next the interaction of the histone chaperones RbAp46 or 48 with histones H3 or H4, then the interaction of EED with the product of PRC2 catalysis, H3K27me3 and the interaction of PRC2 components with a long non coding RNA (ncRNA). The binding specificity could be modulated by the variation in the composition of the PRC2 holoenzyme and posttranslational modifications (PTMs) of its components. The ncRNA is thought to be a major player in the cell-specific recruitment of PRC2 (Figure 10). A large pool of long ncRNA may function to direct the complex to defined target genes and this targeting may not necessarily entail linear base pairing with target sequences, but instead the tertiary structure of the RNA may be the key to specific target gene recognition (Margueron and Reinberg, 2011).

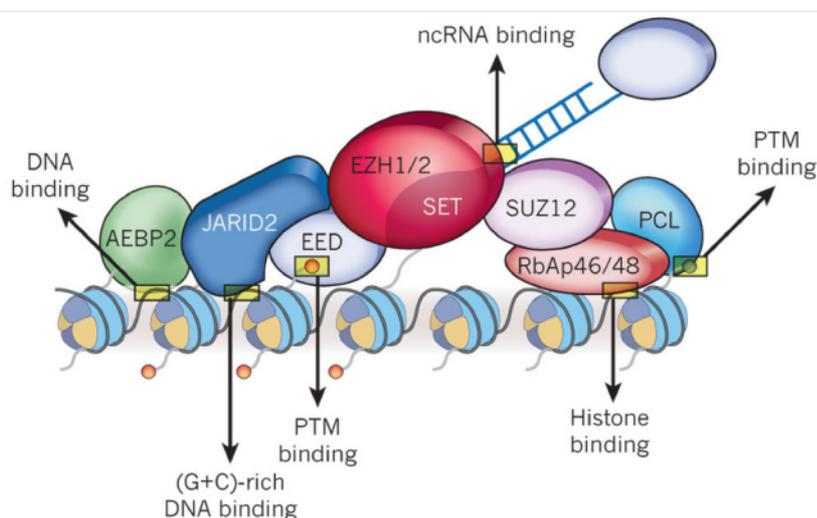


FIGURE 10: Schematic representation of the PRC2 holoenzyme at chromatin. Putative interactions with either DNA or histones that could explain PRC2 recruitment are highlighted. From (Raphaël Margueron and Danny Reinberg, *Nat Rev*, 2011)

PRC2 catalytic unit: EZH2

EZH2 gene located on 7q36 locus, encodes for a protein (~95 kDa), which is the catalytic subunit of the PRC2. EZH2 is a highly conserved histone methyltransferase that methylates lysine 27 of histone 3. It contains conserved regions including cysteine-rich

amino acid stretch that precedes the carboxy-terminal SET domain with HMTase activity (Xiao, 2011). EZH2-mediated methylation is a potential independent mechanism for epigenetic silencing of tumor suppressor genes in cancer. Oncogenic activity of overexpressed *EZH2* has been shown in a wide range of cancers such as those of the prostate and breast (Deb et al., 2013, Yu et al., 2007).

EZH2 has been reported to harbor various heterozygous mutations at tyrosine 641 (Y641) in the C-terminal SET domain (Morin et al., 2010) resulting in a gain of function. Although initially reported to be a loss-of-function mutation, subsequent studies have shown that the Y641 mutation actually results in a gain of function (Yap et al., 2011). These mutants have enhanced catalytic efficiency for dimethylated H3K27me₂, comparing to the wild type showing substrate preference for unmethylated H3K27 and monomethylated H3K27me₁, therefore both the wild type and the Y641 mutants can work together to increase the levels of H3K27me₃. It has been reported that such mutations are found in 7% of follicular lymphomas and 22% of germinal center B-cell and diffuse large B-cell lymphomas (DLBCLs) (Tan et al., 2014).

Unlike in B-cell lymphomas, *EZH2* mutations in myeloid malignancies are inactivating/hypomorphic (Khan et al., 2013). In a recent study, Khan et al. studied the mutational status by Sanger sequencing in myeloid malignancies and found *EZH2* mutations in 8% of cases (n=469 cases examined). In addition, they observed reduced *EZH2* expression in 78% of cases with hemizygous deletion (-7/del7q cases involving *EZH2* locus), and 41% of cases with diploid chromosome 7 (Khan et al., 2013).

Another study suggested that in patients with myeloid disorders, *EZH2* mutations were found most commonly in patients with myelodysplastic/myeloproliferative neoplasms (27 out of 219 individuals, or 12%) and in those with myelofibrosis (4 out of 30 individuals, or 13%) (Ernst et al., 2010).

In addition to its role as a transcriptional repressor, several studies have shown that EZH2 may also function in target gene activation. It has been recently found that a subset of EZH2-bound genes did not bind the PRC2 subunit SUZ12 or display H3K27me₃. Many of these genes were down regulated upon *EZH2* knockdown, suggesting that the role of *EZH2* as an activator was independent of the PRC2 complex (Xu et al., 2012).

They found that the oncogenic function of *EZH2* in resistant prostate cancer cells is independent of its role as a transcriptional repressor, but it involves the ability of *EZH2* to act as a coactivator for critical transcription factors such as androgen receptor.

PRC2 accessory sub-unit: ASXL1

The *Additional sex combs like 1* (*Asxl1*) gene, located on 20q11 locus, is 1 of 3 mammalian homologs of the *Additional sex combs* (*Asx*) gene of *Drosophila*. *Asx* genes are unusual because they belong to both *PcG* genes, which silence their targets, and *trxG* proteins that maintain transcriptional activation. These proteins that are capable of both activation and silencing are grouped in the Enhancer of trithorax and Polycomb (ETP) proteins (Katoh, 2013).

All mammalian ASXL proteins have conserved sequence features: an amino-terminal ASX homology (ASXH) region and a carboxy-terminal plant homeodomain (PHD) domain (Fisher et al., 2010). ASXL1 (~165 kDa) functions autonomously as a transcriptional activator in human cancer cell lines that are retinoic acid (RA) sensitive. It enhances recruitment of RA receptors to their chromatin targets, and in the presence of the ligand RA, it interacts with the activation domain core of RA receptors to increase their activity. However, in other cancer cell lines that are RA resistant, ASXL1 is a co-repressor of retinoic acid receptor (RAR) activity, supporting the dual activator/repressor functions for mammalian ASXL proteins depending on cellular context (Cho et al., 2006).

Asxl1 is expressed in all hematopoietic cell fractions and *Asxl1* knockout mice exhibit defects in frequency of differentiation of lymphoid and myeloid progenitors, but not in multipotent progenitors. In addition no effects have been detected on hematopoietic stem cells, or in peripheral blood. These observations suggest that *Asxl1* is needed for normal hematopoiesis (Fisher et al., 2010).

ASXL1 interacts with components of PRC2, including *EZH2* and *SUZ12*, and it has been shown that *ASXL1* mutations result in loss of PRC2-mediated histone H3

lysine 27 tri-methylation; the re-expression of *ASXL1* promotes H3K27me_{2/3} to partially inhibit the aberrant expression of the *HOXA*-cluster genes in *ASXL1*-null leukemic cells and loss of *ASXL1 in vivo* promotes myeloid leukemogenesis (Abdel-Wahab et al., 2012). It remains unclear whether ASXL1 binds directly to a PRC2 component, however ASXL1 recruits PRC2 to specific genomic loci, where it adds the H3K27me₃ to further recruit PRC1 for transcriptional repression (Katoh, 2013).

It has been widely demonstrated that recurrent somatic *ASXL1* mutations occur in patients with myelodysplastic syndrome, myeloproliferative neoplasms, and acute myeloid leukemia, and these mutations are associated with adverse outcome. These mutations cause truncation of the protein downstream of the ASXH domain with consequent loss or translocation of the PHD domain in leukemic cells (Fisher et al., 2010).

ASXL1 mutations have been identified in 13% of patients with MDSs and 43% of patients with CMML, 7% with primary and 47% with secondary AML (Tefferi, 2010). *ASXL1* was identified as the second most mutated gene after *TET2* in BPDCN patients, reported by Menez et al. They demonstrated that (32%) of a total of 42 BPDCN (samples obtained at diagnosis) studied cases were mutated by WES study (Menezes et al., 2014).

Taken together this evidence raises the possibility that perturbations in the epigenetic modifying machinery play a crucial role in the pathogenesis of BPDCN and similar malignant hematopoietic disorders (Table 1).

While the genetic causes of cancer have been intensively studied, it is becoming evident that a large proportion of cancer susceptibility cannot be attributed to differences in protein-coding sequences. This has been highlighted by genome-wide association studies in cancer, revealing that more than 80% of cancer-associated single nucleotide polymorphisms (SNPs) occur in noncoding regions of the genome (Cheetham et al., 2013). There is emerging evidence indicating that a significant fraction of the genetic etiology of cancer is caused by noncoding regulatory sequences, particularly by long noncoding RNAs (lncRNAs).

Gene	Myeloid malignancy	Mutation frequency, %	Biology	Clinical implications of mutations
TET2	AML	7–23	Normally converts 5-mC to 5-hmC, an intermediate event leading to demethylation. Deletion of Tet2 in vivo results in increased HSC self-renewal and development of myeloid skewing in differentiation.	Worse prognosis in favourable CN-AML, lower incidence of CR. No clear prognostic importance in MDS. Also, no clear prognostic importance in MPNs, although an increased frequency of TET2 mutations has been reported on leukaemic transformation of MPNs.
	MDS	20–25		
	MPN	4–13		
	MDS/MPN overlap	36–58		
IDH1 and IDH2	AML	15–33	Mutants produce 2-HG, which inhibits TET2 (and potentially other enzymes). There seems to be biological and clinical differences between IDH2-R140Q and other mutations of IDH1 and IDH2. The overexpression in primary mouse tissues is linked with an increased LSK compartment and increased KIT expression.	Results on prognostic effects in AML are divergent but the largest studies with the most uniformly treated patients reveal favourable prognostic importance with IDH2-R140Q mutations and no impact of IDH1-R132H or IDH2-R172K mutations on OS for patients with CN-AML. No clear prognostic importance in MDS. Rare mutations in the chronic phase of MPN, for which mutations in IDH1 or IDH2 are linked with leukaemic transformation.
	MDS	3.5		
	MPN	2.5–5		
	MDS/MPN overlap	5–10		
DNMT3A	AML	12–22	De novo methylation of DNA. Mutations are thought to be loss-of-function, although the effect of a recurrent mutation at R882 is not well-clarified. DNMT3A mutations have been linked with HOXA and HOXB overexpression in AML patient samples. Deletion of Dnmt3a in mouse bone marrow results in an expansion of the stem cell pool owing to increased stem-cell self-renewal without development of overt malignancy.	Several studies show that DNMT3A mutations are associated with worsened OS in a cohort of patients with AML, in addition to patients with CN-AML. A single study revealed an adverse prognosis of DNMT3A mutations in MDS. No known prognostic importance, if any, in patients with MPN.
	MDS	8		
	MPN	7–15		
	MDS/MPN overlap	4		
ASXL1	AML	5.2	Unclear if mutations confer a loss or gain of function. Role in mammalian haematopoietic-specific context is not known. Constitutional knockout in mice is associated with partial perinatal lethality, and surviving mice had minimal phenotype.	Worse OS in MDS and AML.
	MDS	14		
	MPN	2–23		
	MDS/MPN overlap	27–49		
EZH2	AML	rare	H3K27 methyltransferase and enzymatic member of PRC2. Affected by loss-of-function mutations in myeloid malignancies. Biological effects of this are unclear as Ezh2 conditional knockout mice had minimal myeloid haematopoietic defects.	Worse OS in MDS and PMF. Mutations are also prevalent in CMML and are associated with adverse outcome.
	MDS	6.4		
	MPN	3–13		
	MDS/MPN overlap	6		
MLL	AML	4–7 (PTD), 10–15 (translocations)	H3K4 methyltransferase. Translocations that replace the methyltransferase domain cause leukaemic transformation and are frequent in infant leukaemias (both myeloid and lymphoid), de novo AML and therapy-associated AML. Oncogenicity of translocations is frequently linked with acquisition of H3K79 methyltransferase activity. PTDs also occur in de novo AML and are linked with increased abundance of H3K4me3 at target genes. Constitutional MLL-PTD expression in vivo results in increased haematopoietic progenitors in vivo but no overt haematological malignancy.	Poor prognosis of MLL-PTD in AML and CN-AML. Translocations at the MLL locus (11q23) are categorized as adverse cytogenetic findings in AML. Often translocated in therapy-associated AML and MDS, particularly after exposure to topoisomerase II inhibitors.
	MDS	Not present in de novo MDS		
	MPN	Not present in MPNs		
	MDS/MPN overlap	Rare		

TABLE 1: The role of mutations in epigenetic regulators in myeloid malignancies. From (Shih et al., *Nat Rev Cancer*, 2012)

2.2.2.7 Emerging epigenetic players: non-coding RNAs

The work presented in this doctoral thesis indicates a critical role for lncRNA in BPDCN pathogenesis. In this setting, I will introduce and develop the importance of non-coding RNAs (ncRNAs) in epigenetic regulation and how deregulation of this function can contribute to cancer progression.

The discoveries over the past decade have highlighted the evidences suggesting that there is an emerging category of molecules playing a crucial role in normal and neoplastic development (Cheetham et al., 2013). These discoveries suggested that

RNA is not only functional as a messenger between DNA and protein but is also involved in the regulation of genome organization and gene expression. These evidences were largely confirmed by analysis that compared transcriptomes with genomes of mammalian species. These data have established that approximately two-thirds of genomic DNA is pervasively transcribed, but only <2% of these RNAs are ultimately translated into proteins (Djebali et al., 2012). This category of RNA is called non-coding RNA.

ncRNAs comprise a diverse group of transcripts including 'housekeeping' ncRNAs (ribosomal RNA, transfer RNA, small nuclear RNA and small nucleolar RNA) and regulatory ncRNAs. Regulatory ncRNAs can be broadly classified according to their sizes in to small ncRNAs (<200 bps, e.g. miRNAs, siRNAs, piRNAs) and large ncRNAs (e.g. lincRNAs, macroRNAs) (Pauli et al., 2011) (Table 2). Some ncRNA can also originate from various gene regulatory elements (enhancers, promoters, transcription start site (TSS) and terminators) and exert divers functions essentially linked to gene expression regulation (Pauli et al., 2011).

Regulatory ncRNAs				
Class according to size	Abbreviation	Full name and comments	Function	Size
small	miRNAs	microRNAs	Gene regulation	18-21 nts
	miRtrons	splicing-dependent intronic microRNAs	Gene regulation	
	endo-siRNAs	endogenous small interfering RNAs	Gene regulation	20-25 nts
	rasiRNAs	repeat-associated small interfering RNAs	Orientation of heterochromatin in the formation of centromeres	24-27 nts
	piRNAs	Piwi-associated RNAs	Regulation of transposon activity and chromatin state	26-30 nts
large	lincRNA	large intergenic non-coding RNAs	Gene expression regulation	> 200 nts
	macroRNAs	lncRNAs, often used for imprinted lncRNAs	Genetic imprinting	up to 400 kb
	ncRNAs-a	activation lncRNAs	cis-activating transcription	> 200 nts

TABLE 2: Regulatory ncRNAs grouped by size. From (Pauli et al., Nat Rev, 2011)

Long non-coding RNA

Long ncRNAs (lncRNAs) comprise heterogeneous class of ncRNAs that are often polyadenylated and devoid of evident open reading frames (ORFs). Many roles are emerging for these RNAs in ribonucleo-protein complexes that regulate various stages of gene expression. Unlike small ncRNAs, lncRNAs can fold into complex secondary and higher

order structures to provide greater potential and versatility for both protein and target recognition (Guttman and Rinn, 2012). Moreover, their flexible and modular scaffold nature enables them to tether protein factors that would not interact or functionally cooperate if they only relied on protein-protein interactions (Tsai et al., 2010).

A specific subgroup of lncRNAs is long intervening noncoding RNAs (lincRNAs, also called long “intergenic” noncoding RNAs), which do not overlap exons of either protein coding or other non-lincRNA types of genes. LincRNAs are stable RNA polymerase II products, and are nearly always capped and polyadenylated, and frequently spliced (Ulitsky and Bartel, 2013).

Mechanistically lncRNAs can be divided into two distinct functional groups concerning their sub-cellular localization, nuclear and cytoplasmic lncRNAs. Cytoplasmic lncRNAs often show sequence complementarity with transcripts that originate from either the same chromosomal locus or independent loci. These mechanisms permit for example the modulation of mRNA stability positively (Kretz et al., 2013) or negatively (Gong and Maquat, 2011), or act by competing with endogenous RNAs through their miRNA recognition motifs (Cesana et al., 2011). The recognition of the target by base pairing allows them specially to modulate translational control of protein expression (Carrieri et al., 2012, Yoon et al., 2012). In contrast, numerous evidences indicate that most nuclear lncRNAs function as gene transcription modulators. In the following section I will develop more specifically the functional mechanisms by which nuclear lncRNAs regulate gene expression in cells.

Mechanisms of function of nuclear long non-coding RNA

Studies of dosage compensation, imprinting, and homeotic gene expression suggest that individual lncRNAs can function as the interface or scaffold between DNA and specific chromatin remodeling factors (Ponting et al., 2009). Nuclear lncRNA can act in *cis*, or in *trans* and activate or repress gene expression via the recruitment of chromatin modifying complexes (Figure 11). It has been shown that at least 38% of lncRNAs present in several human tissues bind to the polycomb repressive complex 2 or other

repressive chromatin-modifying proteins (Khalil et al., 2009) and the rest of them bind to trithorax chromatin-activating complexes and/or activated chromatin (Dinger et al., 2008), some of these mechanisms will be further described both in physiological and pathological context in the next section.

Physiological roles of lncRNA expression in gene regulation

X-chromosome inactivation

Dosage compensation is among the first and best characterized example of lncRNAs required for normal development. They are responsible for the formation of silenced chromatin and thus the production of monoallelic expression of specific genes in mammals.

X chromosome inactivation (XCI) is a compensation mechanism for dosage differences between males and female, it renders all cells functionally monosomic for the X chromosome. XCI is coordinated by the X-inactivation center (Xic), which controls most of the steps of this process, including X chromosome counting, random X chromosome choice, and the initiation of silencing along ~1000 genes of the X. In mammals *Xist* (X-inactive specific transcript) is a lncRNA that is directly involved in the formation of repressive chromatin. *Xist* produces a 17-20 kb RNA that decorates the X chromosome during the initiation of XCI (Clemson et al., 1996). *Xist* RNA directs chromatin and transcriptional change by binding Polycomb repressive complex 2 (PRC2), which is the epigenetic complex responsible for trimethylation of histone H3 at lysine 27 (H3K27me3) (Figure 11 Aa) from the nucleation center that consists of three binding sites for the transcription factor YY1 (Yin Yong 1, transcriptional repressor protein), a protein bound only to the Xi (X-inactive) allele. PRC2 spreads initially to ~150 strong binding sites along the future Xi. As XCI proceeds, the coating of the future Xi by *Xist* RNA correlates with recruitment of 3,000-4,000 moderate polycomb sites. The moderate sites cluster around strong sites facilitates the spreading of H3K27me3 in a graded concentration relative to strong sites (Lee and Bartolomei, 2013).

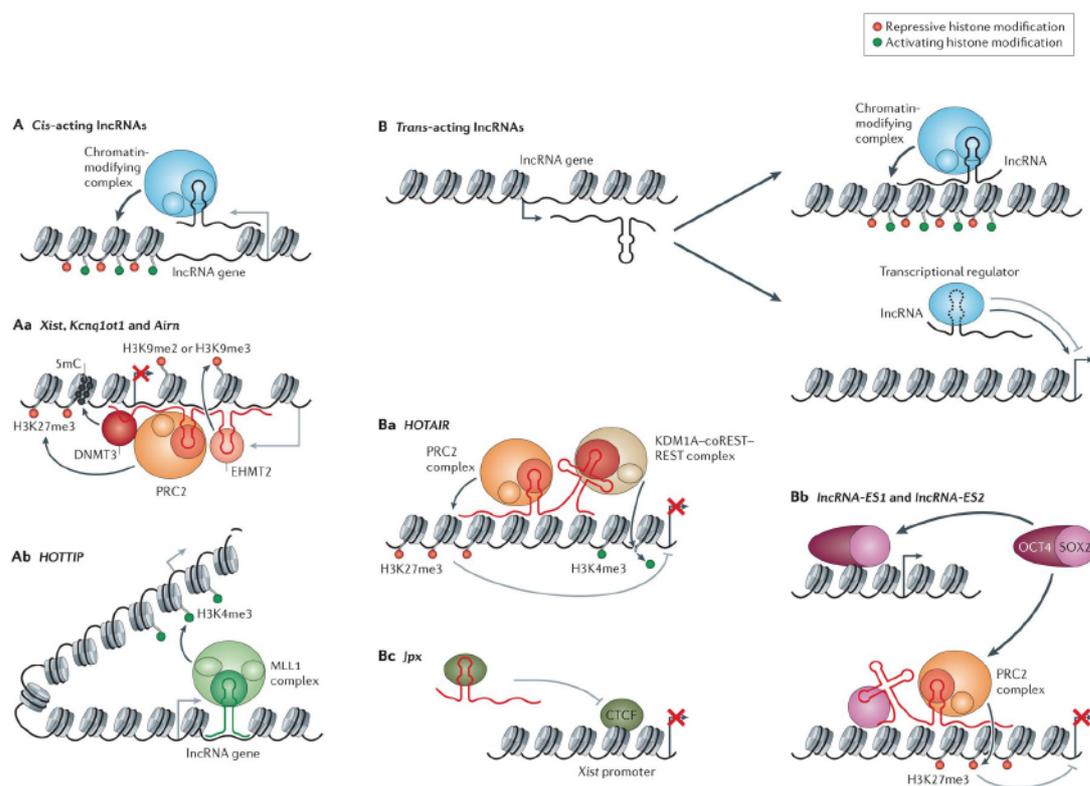


FIGURE 11: Models for nuclear lncRNA function: Long non-coding RNAs (lncRNAs) regulating transcription in cis (part A) and in trans (part B). Aa) *cis*-acting lncRNA examples are; X-inactive specific transcript (Xist), Kcnq1 overlapping transcript 1 (Kcnq1ot1) and Airn (antisense Igf2r (insulin-like growth factor 2 receptor) RNA). These lncRNAs induce the formation of repressive chromatin through the recruitment of DNA methyltransferase 3 (DNMT3), which induces DNA methylation; Polycomb repressive complex 2 (PRC2), which produces histone H3 lysine 27 trimethylation (H3K27me3); and histone lysine N-methyltransferase EHMT2, which is responsible for producing H3K9me2 and H3K9me3 (Lee and Bartolomei, 2013). Ab) HOXA distal transcript antisense RNA (HOTTIP) functions through the recruitment of the mixed lineage leukemia 1 (MLL1) complex, which drives the formation of the activating H3K4me3 mark (Wang et al., 2011). Ba — HOXA transcript antisense RNA (HOTAIR) is a *trans*-acting regulator of the HOXD genes (Rinn et al., 2007). It is characterized by a modular scaffold structure that allows the recruitment of two distinct repressive complexes, PRC2 and the H3K4 demethylating complex KDM1A-coREST-REST (lysine-specific histone demethylase 1A-REST corepressor 1-RE1-silencing transcription factor) on the same genomic region (Tsai et al., 2010). Bb — The pluripotency RNAs lncRNA-ES1 and lncRNA-ES2 associate with both PRC2 and the transcription factor sex-determining region Y-box 2 (SOX2), which suggests that these lncRNAs control embryonic stem cell pluripotency by silencing SOX2-bound developmental genes (Ng et al., 2012); this function is alternative to OCT4 -and SOX2-dependent activation of pluripotency genes. Bc — The lncRNA Jpx (Jpx transcript, Xist activator) that binds to the transcriptional repressor CTCF inhibits its binding to the Xist promoter, thus activating Xist transcription (Sun et al., 2013). From (Fatica and Bozoni, Nat Rev Genet, 2014)

Xist expression itself is controlled by other lncRNAs in both a positive and a negative manner. *Tsix* is one of the best-characterized *Xist* regulators, which is a natural antisense non-coding transcript. *Tsix* counteracts *Xist* expression by inducing repressive epigenetic modifications at the *Xist* promoter (Lee and Bartolomei, 2013). *Xist* activation also requires the lncRNA *Jpx* (Tian et al., 2010), which induces *Xist* transcription through the sequestration of transcriptional repressor CTCF (Figure 11 Bc) (Fatica and Bozzoni, 2014).

Genomic imprinting

Genomic imprinting is briefly defined as parental-specific gene expression. Imprinted genes are generally associated in clusters and are epigenetically marked in different and sex-dependent ways during male and female gametogenesis and embryonic development. Similarly, genomic imprinting is regulated by *cis*-acting imprinting control regions (ICRs) that influence allelic expression across long distances.

Imprinted genes encode various species of ncRNAs, including lncRNAs (in general longer than 100 kb). In many cases, these lncRNAs bind to imprinted regions and are directly involved in silencing. Two best-characterized examples are the lncRNAs *Kcnq1* overlapping transcript 1 (*Kcnq1ot1*) and *Airn* (antisense *Igf2r* (insulin-like growth factor 2 receptor) RNA); these lncRNAs are paternally expressed, and function by repressing flanking protein-coding genes in *cis* (Lee and Bartolomei, 2013). During embryonic development, *Kcnq1ot1* establishes and maintains repressive DNA methylation on surrounding genes, whereas, in the placenta, it functions by recruiting the repressive histone modifiers PRC2 and euchromatic histone-lysine N-methyltransferase 2 (EHMT2) (also known as G9a) on genes that are located further away from the imprinted region (Mancini-Dinardo et al., 2006).

The other example is *Airn*, which silences the paternal *Igf2r* allele in *cis*, whereas the maternal *Igf2r* allele remains expressed. In embryo, *Airn* silences paternal *Igf2r* through a mechanism that does not require a stable RNA product but that is based

on continuous Airn transcription, which interferes with the recruitment of RNA polymerase II (Latos et al., 2012), but in placenta, mature Airn recruits EHMT2 to induce the formation of repressive chromatin (Sleutels et al., 2002) (Figure 11 Aa).

Regulation of *HOX* genes

In mammals, there are 39 *HOX* genes that are grouped in four clusters (*HOXA*, *HOXB*, *HOXC* and *HOXD*), which allow precise spatiotemporal expression of evolutionary conserved family of transcription factors. In addition to protein-coding genes, these clusters produce lncRNAs that show similar spatiotemporal windows of expression to their flanking protein-coding genes and it has been shown that they are implicated in the regulation of *HOX* genes (Rinn et al., 2007).

HOTTIP is the *cis*-acting antisense transcript of *HOXA* locus. HOTTIP regulates *HOXA* expression by interacting with the activating H3K4 methyl transferase, mixed lineage leukemia 1 (MLL1) complex, and by the formation of chromatin loops that connect distally expressed HOTTIP transcripts with various *HOXA* gene promoters (Wang et al., 2011) (Figure 11 Ab).

HOTAIR (*HOXC* transcript antisense RNA) was one of the first *trans*-acting lncRNAs to be identified and it acts as a repressor of the *HOXD* cluster, which is located on a different chromosome. HOTAIR acts as a molecular scaffold with two known chromatin modification complexes; the 5' region of this lncRNA binds to the PRC2 complex responsible for H3K27 methylation and the 3' region binds to LSD1, which mediates enzymatic demethylation of H3K4 (Tsai et al., 2010) (Figure 11 Ba).

Long noncoding RNA in cancer development

As for protein coding genes, ncRNAs contribute to the regulatory networks that participate to cancer development. Their implication in cancer development has been detected by various techniques including RNA-sequencing (RNA-seq), next generation sequencing, and methylation analysis (Trapnell et al., 2010). These approaches have

led to the identification of several lncRNAs whose expression and the epigenetic state of their coding genes were associated with cancer cells or tissues. Proteins, controlling chromatin organization including polycomb repressor complexes, PRC1 and PRC2, and members of the trithorax family constitute key players in the molecular pathogenesis of cancer. As mentioned before, many evidences suggest that a major role of lncRNAs is to guide the site-specific chromatin-modifying complexes to target genes and contribute to the epigenetic modification of these genes (Mattick and Gagen, 2001). Recent studies have linked the miss-expression of well-characterized lncRNAs to diverse cancers, such as HOTAIR in breast cancer, AINRIL and SChLAP1 in prostate cancer (Gupta et al., 2010, Kotake et al., 2011, Prensner et al., 2013).

HOTAIR is one of the first lncRNA discovered to be involved in tumorigenesis. Its expression is up regulated in primary tumors and up to 2000-fold in metastatic breast cancer (Gupta et al., 2010). Overexpression of HOTAIR in epithelial cancer cells induces genome-wide re-targeting of PRC2 to an occupancy pattern more resembling embryonic fibroblasts, resulting to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a PRC2 dependent manner (Gupta et al., 2010).

Prostate cancer is another example in which lncRNAs has been implicated in disease development and progression. As an example, SChLAP, a lncRNA identified by RNA-seq in prostate cancer, shows high level expression in aggressive forms is predictive of poor outcomes, and metastasis. *SChLAP1* appears to mediate this function by antagonizing the genome-wide localization and regulatory functions of the SWI/SNF chromatin-modifying complex (Prensner et al., 2013). SWI/SNF complexes interact with transcription factors, co-activators, and corepressors and are capable of mobilizing nucleosomes at target promoters and enhancers to modulate gene expression (Tolstorukov et al., 2013). Loss of SWI/SNF complex functionality promotes cancer progression, and it has been shown that multiple SWI/SNF components are somatically inactivated in cancer (Reisman et al., 2009).

AINRIL (antisense non-coding RNA in the *INK4* locus) is a 3.8 kb-long non-coding RNA expressed in the opposite direction from *INK4A-ARF-INK4B* gene cluster

and is important for expression of the protein-coding genes in cis. *ANRIL* activates two polycomb repressor complexes, PRC1 and PRC2 by direct binding to chromobox 7 (CBX 7) and SUZ12, members of PRC1 and PRC2, respectively (Kotake et al., 2011, Yap et al., 2010). This results in silencing the *INK4b-ARF-INK4a* locus encoding tumor suppressors p15INK4b, p14ARF, and p16INK4a, which are usually altered in an estimated 30-40% of human tumors. These genes are implicated in cell cycle inhibition, senescence and stress-induced apoptosis.

Selective binding of lncRNAs, such as *HOTAIRE*, *ANRIL* and *SChLAP1* with chromatin modifying complexes and execute histone modifications at specific loci thus strongly supports the idea that lncRNAs may function as ideal epigenetic regulators in cells (Kotake et al., 2011). LncRNAs are also implicated in other cellular functions such as DNA repair in response to genotoxic stress (Prensner et al., 2014), but to our knowledge the main function of lncRNAs currently is the regulation of gene expression, therefore it is obvious that their maintained expression remains critical in order to keep cellular homeostasis and prevent the neoplastic transformation.

2.2.3 Gene expression profiling of BPDCN - insights to molecular pathogenesis

Besides chromosomal abnormality and mutational analysis, gene expression profiling (GEP) provides important insights, which cannot be explored by cytogenetic and mutational studies. For example Dijkman et al performed GEP analysis on 5 BPDCN and 3 cutaneous-AML samples and have identified high-level expression of Notch signaling genes (*HES6*, *RUNX2*). Moreover, despite the mono-allelic deletion of *FLT3* gene, increased expression of *FLT3* oncogene was also observed in BPDCN, coincident with expression of both myeloid and lymphoid specific gene expression (Dijkman et al., 2007).

In a second study, a global GEP analysis on 6 BPDCN cases, in comparison with normal pDCs, myeloid and lymphoid precursor cells has demonstrated predominant expression of myeloid lineage genes and resting pDCs. This work also discovered evidence of an aberrant activation of the NF- κ B pathway in BPDCN, which was

confirmed by Immunohistochemistry (IHC) by showing the nuclear localization of key components of NF- κ complex (c-Rel and p50) in BPDCN-derived cell line and BPDCN cases, in contrast to normal pDCs, which presented a cytoplasmic staining. This was associated to sensitivity to NF- κ B inhibition and thus suggestive of a novel therapeutic strategy in BPDCN (Sapienza et al., 2014).

Chapter 3

Clinical management of BPDCN

Given BPDCNs rarity and only recent recognition as a distinct clinico-pathological entity, no standardized therapeutic approach has been established for BPDCN and the optimal therapy remains to be defined. Current practice considers acute myeloid leukemia-like or acute lymphoblastic leukemia (ALL)-like regimens acceptable for induction treatment.

Patients usually respond well to initial chemotherapy, with complete response rates of 47% to 86%. However, the disease often relapses, and the relapsed cases are typically resistant to the previously used chemotherapeutic agents (Fachetti, 2008, Feuillard et al., 2002). Outcomes are more favorable in cases that lack cutaneous disease at presentation, although a comparison of cutaneous and non-cutaneous cases might be confounded by differences in treatment regimens. Conversely, patients with isolated cutaneous lesions survive longer (Alayed et al., 2013, Shi and Wang, 2014).

These short-lived responses, with second remissions that are difficult to achieve, underscore the need to consider hematopoietic cell transplantation early in the disease course (Kharfan-Dabaja et al., 2013) as long lasting remissions have been documented in sporadic cases, usually occurring in young patients who have been treated with acute leukemia-type induction therapy, followed by allogeneic stem cell transplantation in first complete remission (Fachetti, 2008).

Durable remissions have been observed even in elderly patients, followed by allogeneic stem cell transplant from matched related or unrelated donors after high-dose chemotherapy; in a retrospective study on 39 patients with BPDCN who underwent allogeneic stem cell transplantation (allo-SCT), $n = 34$, or autologous stem cell transplantation (auto-SCT), $n = 5$. The 3-year cumulative incidence of relapse, disease-free survival, and overall survival of these patients was 32%, 33%, and 41%, respectively (Roos-Weil et al., 2013).

It is noteworthy that although the disease is recognized among acute myeloid leukemias and acute myeloid leukemia-like regimens are often adopted, recent reports have suggested a potential role for ALL-like protocols (Pagano et al., 2013, Piccaluga et al., 2012, Pileri et al., 2012). Recently, in a case report study, it has been demonstrated that using a combined ALL-like and AML-like treatment protocols followed by cord blood stem cell transplant have achieved a complete remission (10 months since diagnosis) (Ramanathan et al., 2013).

Since AML, ALL and lymphoma type therapies actually are used for BPDCN treatment, therefore these therapies are explained in the following section.

3.1 Conventional AML type therapy

Traditionally AML treatment involves two phases: 1) remission induction therapy, 2) consolidation, or post-remission, therapy and then maintenance therapy. Remission induction is designed to bring about complete remission, and consolidation therapy is designed to kill any remaining leukemia cells. AML is usually treated with two or three anti-cancer drugs (combination chemotherapy): the initial treatment applied to AML consists of cytosine arabinoside (ara-C or Cytarabine) (is a nucleoside analogue that incorporates DNA and induces apoptosis) combined with anthracycline (DNA and RNA intercalating that prevents DNA replication and transcription). Consolidation therapy includes one to two courses of high-dose chemotherapy using only ara-C, or a hematopoietic cell (stem cell or bone marrow) transplant (Roboz, 2012).

Most patients will experience a relapse of their disease without this additional therapy. Approximately 70%-80% of patients <60 years of age will achieve complete remission, but most ultimately relapse and overall survival is only 40%-45% at 5 years (Roboz, 2011).

3.2 Conventional ALL type therapy

Glucocorticoids (GCs) particularly prednisone and dexamethasone were among the first drugs used in the treatment of acute lymphoblastic leukemia (ALL) and have remained essential components of therapy. The most commonly used steroids are dexamethasone and prednisone. ALL has a higher incidence rate in children than in adults with a median age of 39 years. Treatment usually includes multi-agent chemotherapy with induction, consolidation, and maintenance.

Although a significant number of ALL patients respond well to GCs, some reveal primary GC resistance; approximately 10% of children with ALL will respond poorly to GCs, and GC resistance is associated with a significantly inferior outcome (Bhadri et al., 2012). In addition, those sensitive to GCs almost exclusively develop secondary resistance after prolonged GC therapy (Roychowdhury and Talpaz, 2011). Without GC sensitivity the leukemic cells are no longer induced into apoptosis by the treatment (Bhadri et al., 2012). Resistance to GCs in ALL has been extensively studied but remains very poorly understood at the mechanistic level. (Bhadri et al., 2012).

3.3 Conventional Lymphoma type therapy

As BPDCN had been classified as Natural killer (NK)/T-cell lymphomas and NK-cell leukemias, T-cell lymphoma, lymphoma type therapy has been used in BPDCN clinical management. These lymphomas are aggressive malignancies in which neoplastic cells present surface marks such as CD56+ and with cytoplasmic germ line T-cell receptor gene. They occur commonly in the nasal and upper aerodigestive with occasional skin

lesions. Concomitant/sequential chemotherapy and radiotherapy is standard treatment; L-asparaginase (a hydrolase that catalyzes the L-asparagine to an endogenous amino acid necessary for the function of some neoplastic cells and resulting in inhibition of RNA and DNA synthesis with the subsequent blastic cell apoptosis) has been incorporated into several regimens as it was found to be effective for relapsed/refractory NK/T-cell lymphomas. One of the most intensive regimens used currently against NK/T cell lymphoma is a chemotherapy protocol called “SMILE” that consisted of steroid (dexamethasone), methotrexate, ifosfamide (causing interstrand cross-links (ICLs) and thus induces apoptosis), L-asparaginase and etoposide (Tse and Kwong, 2013).

Etoposide is a potent anti-tumor drug used as an apoptosis-inducing chemotherapy agent that belongs to the class of topoisomerase poisons. It has the ability to stabilize the covalent complex between DNA topoisomerase II (Topo II) and DNA, resulting in a high level of DNA damage. Topo II is an essential enzyme that controls the topology of DNA by passing an intact double helix through a transient double-stranded DNA break in an ATP-dependent reaction which eventually leads to apoptosis (Montecucco and Biamonti, 2007).

There are other lymphoma-type protocols that are used in BPDCN containing multiagent chemotherapy, such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), or hyper-CVAD (combination of course A: cyclophosphamide, vincristine, doxorubicin; and course B: methotrexate and cytarabine, in an alternating fashion). These are effective and have been shown to be superior to AML type therapy (Pagano et al., 2013).

Glucocorticoids receptor signaling

Because of the relevance of Glucocorticoid receptor (GCR) signaling to the present work, an overview of GCR signaling is given below. It should be noted that intense efforts are currently underway to derive novel GCR agonists and antagonists and for novel synergy particularly with epidrugs. Briefly, Glucocorticoids (GCs) are a class of

steroid hormones that regulate a wide range of physiologic functions and play an important role in the maintenance of basal, stress-related homeostasis and they are involved in most of cellular regulation networks. It is worth noting that around 20% of the genes expressed in human leukocytes are regulated positively or negatively by glucocorticoids (Nicolaidis et al., 2010).

The human (h)GCR belongs to the steroid/thyroid/retinoic acid, superfamily of transcription factor proteins and functions as a ligand-dependent transcription factor. The hGCR gene is located on chromosome 5(5q31) and consists of nine exons. Two highly homologous isoforms of GCR are found, termed α and β with 97 and 94 kDa, molecular weights respectively. There is conflicting evidence that GCR α is the functional isomer, whereas GCR β does not bind GC, is transcriptionally inactive and potentially functions as a dominant negative inhibitor of GCR activity (Bhadri et al., 2012). The human GCR is composed of an N-terminal domain, containing the transactivation domain termed activation function (AF)-1, a DNA-binding domain and a hinge region and the ligand-binding domain (Figure 12). The transactivation domain plays an important role in the interaction of the receptor with molecules necessary for the initiation of transcription, such as co-activators, chromatin modulators and basal transcription factors, including RNA-polymerase II, TATA-binding protein (TBP). The DNA-binding domain (DBD) contains two zinc finger motifs through which the GCR binds to specific DNA sequences (glucocorticoid-response elements (GREs)) in the promoter regions of target genes. The DBD also contains sequences important for receptor dimerization and nuclear translocation.

The hinge region is a flexible region between the DNA- and ligand-binding domains, which is involved in its dimerization. The ligand-binding domain (LBD) of the GCR corresponds to amino acids 481-777, binds to glucocorticoids and is implicated in the ligand-induced activation of GCR. The LBD also contains a second transactivation domain or AF-2, which is important for receptor dimerization, nuclear translocation, binding to the heat shock proteins (Nicolaidis et al., 2010). The glucocorticoid receptor protein is subjected to post-translational modifications, which impact on the glucocorticoid receptor function (McMaster and Ray, 2008) (Figure 12).

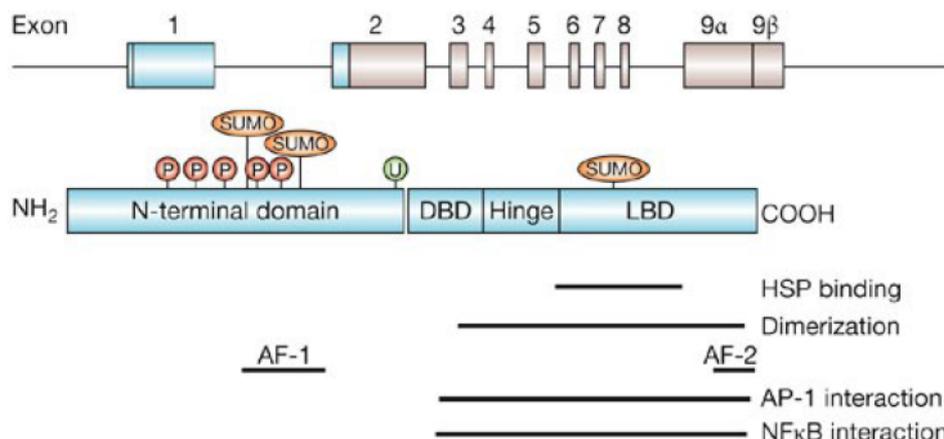


FIGURE 12: Structure of the glucocorticoid receptor; the coding regions are indicated by gray boxes encoded within nine exons; is. The N-terminal domain contains the major transactivation function domain, AF-1. The DBD consists of two α -helices, which coordinate two zinc atoms. This domain is important for dimerization, nuclear translocation, and binding glucocorticoid-response elements and subsequent transactivation. The LBD undergoes conformational change when it binds ligand, enabling interaction with coregulator proteins. Additional functional domains are indicated by the thick horizontal lines marked beneath the structure: the HSP-binding domain; the homodimerization domain; the major transactivation domains AF-1 and AF-2; and the region that functionally interacts with AP-1 and NF κ B. Abbreviations: AF-1, hormone-independent transactivation function domain; AF-2, transactivation domain; AP-1, adapter protein complex 1; DBD, DNA-binding domain; HSP, heat-shock protein; LBD, ligand-binding domain; NF κ B, nuclear factor B; P, sites of phosphorylation on serine or threonine residues; SUMO, sites of SUMOylation (post-translational modification mediated by small, ubiquitin-related modifier proteins); U, site of ubiquitination (post-translational modification mediated by ubiquitin). From (McMaster and Ray, *Nat Clin Pract Endocrinol Metab*, 2008)

Nucleocytoplasmic translocation of GCR

In the absence of ligand, GCR can be found mostly in the cytoplasm as part of a heterooligomeric complex, which contains chaperone heat shock proteins (HSPs) 90, 70 and 50, immunophilins and other proteins. HSP90 regulates cytoplasmic retention of GCR by exposing the ligand-binding site and masking the two nuclear localization sequences (NLS), NL1 and NL2, which are located respectively adjacent to the DBD and in the LBD of the receptor. Upon ligand-induced activation, the receptor undergoes a conformational change that results in dissociation from this multiprotein complex and translocation into the nucleus (Pratt, 1993).

Within the nucleus, the receptor forms a homodimer to GREs in the promoter

regions of target genes, or it interacts as a monomer with other transcription factors protein-1 (AP-1), nuclear factor- κ B (NF- κ B), p53 and signal transducers and activators of transcription (STATs) and regulates gene expression (Nicolaidis et al., 2010). Some of these factors result in Glucocorticoid dependent-lympholytic actions and that's the reason why they are included in many therapeutic regimens for the treatment of various forms of leukemia and lymphoma. As mentioned earlier, significant number of acute lymphoblastic leukemia patients respond well to GC treatment during initial phases, although prolonged treatments sometimes results in steroid-resistance (Hillmann et al., 2000).

3.4 Emerging therapeutic strategies

As discussed above, there is currently no consensus on the optimal treatment for BPDCN. Radiotherapy or local chemotherapy is inefficient and only patients receiving systemic polychemotherapy regimens are likely to achieve complete remission, even if responses are short and mostly followed by drug resistance and relapse in most patients. Molecular profiling in BPDCN suggests that a number of novel targeted agents might be useful in this disease.

3.4.1 Inhibitors of signaling pathways

3.4.1.1 IL3 receptor pathway

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 are members of a small family of cytokines that regulate the production and activities of many hematopoietic cells. Their activities are mediated through binding to the few but high affinity membrane receptors.

IL-3 is expressed mostly by activated T lymphocytes and mast cells and is important for the regulation of mast cells function. One of the biologic activities of IL-3 is

exerted at the level of the progenitor compartment, where this cytokine stimulates the survival and proliferation of multipotent cells (Hercus et al., 2013).

It has been observed that interleukin-3 receptor α (IL3R α) (also known as CD123) is over expressed in progenitor cells from patients with AML, and blast cells from patients with AML proliferate in response to IL-3 (Jordan et al., 2000). IL3R α overexpression is strongly correlated with short patient survival (Vergez et al., 2011). High IL3R α surface expression is ubiquitous in BPDCN blasts but negligible in normal hematopoietic stem cells and BPDCN blasts *in vitro* require IL3 supplementation for growth and survival (Chaperot et al., 2001, Jordan et al., 2000).

In a recent study Frankel and colleagues used SL-401 protein (a recombinant fusion protein composed of the catalytic and translocation domains of diphtheria toxin (DT) fused via a Met-His linker to IL3) (Frankel et al., 2000) as a targeted therapy for BPDCN on a cohort of patients (Frankel et al., 2014). The IL3 domain of SL-401, still capable of binding to its natural receptor, is then internalized, leading to translocation of the DT fragment to the cytosol. SL-401 inhibits protein synthesis by ADP ribosylation of elongation factor 2. They demonstrated that 7 of 9 evaluable (78%) BPDCN patients had major responses including 5 complete responses and 2 partial responses after a single course of SL-401. The median duration of responses was 5 months (range, 1- 20+ months) (Frankel et al., 2014).

3.4.1.2 NF- κ B pathway

Nuclear factor (NF)- κ B is a family of transcription factors that comprises diverse members: p50, p65/RelA, c-Rel, p52, and RelB (Wan and Lenardo, 2009). These transcription factors are implicated in the control of many different cellular mechanisms such as the innate- and adaptive-immune responses and programmed cell death (apoptosis). They can both induce and repress gene expression by binding to promoters and enhancers of DNA sequences known as κ B elements. NF- κ B is normally sequestered in the cell's cytoplasm by a protein called Inhibitor of NF- κ B (I κ B). Numerous signaling pathways induce the activation of NF- κ B during which I κ Bs get phosphorylated and then ubiquitinated, resulting in I κ B degradation by proteasome and the activation

of NF- κ B. In the absence of I κ Bs, NF- κ B is free and can translocate to the nucleus (Perkins, 2007).

As mentioned earlier in a recent study by Sapienza et al., gene expression profiling (GEP) and functional tests show a preclinical rationale for NF κ B pathway inhibitors (Bortezomib) in BPDCN.

Bortezomib is a well-known proteasome inhibitor currently employed in the treatment of multiple myeloma and some non-Hodgkin lymphomas and block proteasome degradation and thus prevents the NF- κ B activation (Sapienza et al., 2014). Bortezomib has been already demonstrated to be very effective and induce rapidly BPDCN cell death in *ex-vivo* experiments (Hirai et al., 2011).

3.4.2 Epidrugs

As explained in previous sections, gene mutations impinging on epigenome modifiers are recurrent in BPDCN (*TET2* mutations, *ASXL1* and *MLL* alterations) Epigenetic therapy can at least in theory counter the ensuing epigenetic disturbances in BPDCN and should show some anti-leukemic activity. Gene derepression consequent to loss of PRC2 function could be countered by HAT (histone acetyl transferase inhibitors) or even BET (bromo and extra terminal domain) protein inhibitors while as mentioned earlier DNMT inhibitors could counter DNA hypermethylation phenotypes associated to *TET2* mutation. More detailed functional studies including epigenome profiling (ChIP-seq and DNA methylation profiling / sequencing) will be required to determine the best strategies and which patients are most likely to benefit from epidrug therapy.

3.4.2.1 Hypomethylating agent 5-Azacytidine

As mentioned earlier partial or complete chromosomal losses and mutations are common in myeloid malignancies. But loss of gene function can also result from epigenetic transcriptional silencing through methylation of promoter-associated CpG

islands and/or post-translational deacetylation of histones (Haase et al., 2007). Aberrant DNA methylation has been described in cancer cells, and it is hypothesized that azanucleoside therapy induces DNA hypomethylation and re-expression of aberrantly silenced genes particularly tumor suppressor genes in patients with these disorders (Figure 13).

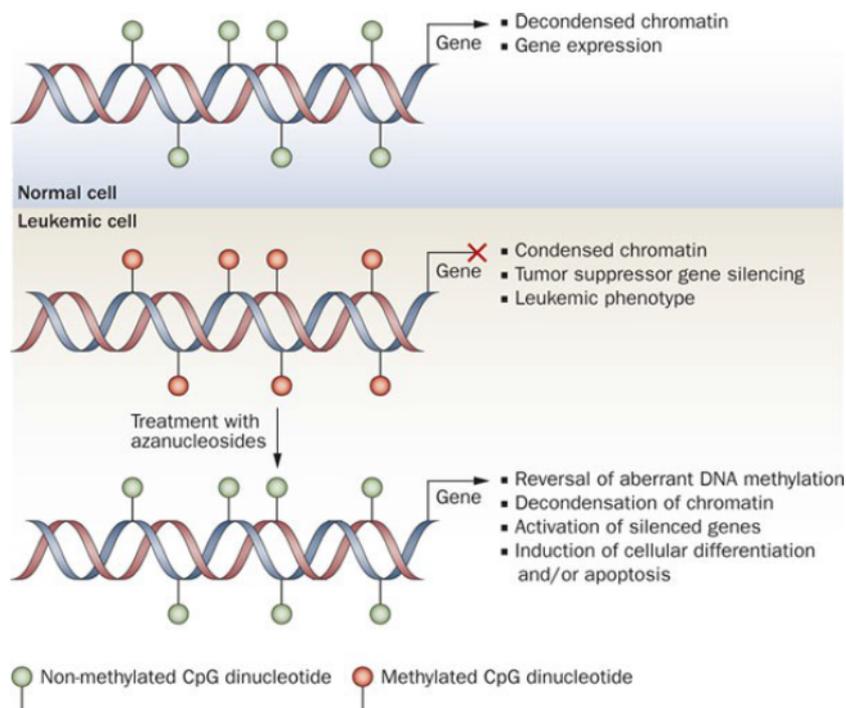


FIGURE 13: Proposed mechanism of action of Azanucleosides. From (Quintas-Cardama et al., *Nat Rev Clin Oncol*, 2010)

Azanucleosides (such as 5-azacitidine and decitabine) are cytidine analogues that incorporate into DNA or RNA and form covalent complexes with DNA methyltransferases resulting in the depletion of active enzymes. Over the successive rounds of cell replication, exposure to hypomethylating agents leads to chromatin decondensation and re-expression of silenced genes. 5-azacitidine therapies have been shown, prolonged survival in patients with MDS (Quintas-Cardama et al., 2010).

Laribi et al. have recently treated two BPDCN cases with 5-azacytidine, both patients showed excellent responses of their skin lesions after one cycle of chemotherapy, and their hematological disease was stabilized, however they passed away for other

medical complications (Laribi et al., 2014). This and similar studies highlight the possible beneficial effects of 5-Aza as a part of the course of treatment, and open new therapeutic opportunities for BPDCN patients.

3.4.2.2 BET bromodomain inhibitors

The studies of epigenetic machinery and its deregulation have led to the development and use of a wide range of modulatory molecules directed not only at chromatin enzymes (histone acetyltransferases, histone deacetylases, histone methyltransferases, histone demethylases and DNA methyltransferases) but also toward the emerging class of chromatin-associated proteins (Filippakopoulos et al., 2010).

One of these chromatin-associated proteins is the bromodomain and extraterminal domain (BET) family of adaptor proteins. This family is comprised of Brd2 (~110 kDa), Brd3 (~80 kDa), Brd4 (~152 kDa), and Brdt (~108 kDa), which performs diverse roles in regulating the transcription by RNA polymerase II (Pol II). BET family proteins are involved in transcription regulation and cell growth and are also implicated in cancer. Every member of BET family proteins possesses two conserved N-terminal bromodomains (BD1 and BD2), which recognize and interact with acetylated lysine residues on histone tails and other nuclear proteins. The recent discovery of potent and highly specific inhibitors for the BET proteins has stimulated intensive research activity particularly in oncology (Filippakopoulos and Knapp, 2014).

There are two independent groups of small-molecule inhibitors of BET bromodomains, called JQ1 and I-BET, which have a similar chemical structure and mode of target inhibition (Filippakopoulos and Knapp, 2014). JQ1 is the first selective inhibitor of BET family proteins and can bind the lysine-binding pocket of bromodomain and show shape complementarity (Filippakopoulos et al., 2010).

It has been demonstrated that BET bromodomains play an important role in supporting the transcription of known oncogenes (*MYC* and *BCL2*) in leukemia and lymphoma (Chapuy et al., 2013, Dawson et al., 2011, Zuber et al., 2011). Filippakopoulos and colleagues suggested that BET inhibition presented an anti-tumor effect by at least

two complementary activities: a specific effect on high BRD4 loaded enhancers (super-enhancers) and a more general suppression of transcription at E2F- and MYC-driven target genes which are under the control of such enhancers, for example in a diffuse large B cell lymphoma model. Thus, the E2F/MYC pathway effect is combined with massive depletion of proteins driven by BRD4-overloaded enhancers, preventing cell cycle progression and leading to growth arrest. These super-enhancers prove particularly sensitive to bromodomain inhibition, explaining the selective effect of BET inhibitors on oncogenic and lineage-specific transcriptional circuits (Chapuy et al., 2013).

JQ1 displays also antitumor effects in human xenograft models of NUT midline carcinoma, inducing cancer cell differentiation and growth arrest in these cells (Filippakopoulos et al., 2010). Using an RNAi screen and JQ1 simultaneously identified BRD4 as a strong therapeutic target and associated JQ1 with robust antileukemic effects *in vitro* and *in vivo* models of AML, accompanied by terminal myeloid differentiation and elimination of leukemia stem cells (Zuber et al., 2011). It is noteworthy that JQ1-mediated BET inhibition and consequent antitumor activity are partly due to *MYC* suppression through disruption of the ability of BET proteins to bind *MYC* locus (Zuber et al., 2011).

It has been also been demonstrated that *in vitro* treatment of lymphoma B cells with JQ1, can inhibit the aggressive attitude of MYC-over-expressing tumors and increase the sensitivity of human lymphoma B cells to Rituximab (Emadali et al., 2013).

Scientific arguments

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) (also termed Blastic natural killer (NK) cell lymphoma or agranular CD4⁺/CD56⁺ hematodermic neoplasm) is a recently described entity, with the first case reported in 1994 (Adachi et al., 1994). It is a rare, clinically aggressive hematologic malignancy with a median overall survival (OS) of 12-14 months and frequent relapses (Fachetti, 2008). Chaperot and colleagues were the first to report and recognize that this disease is a malignancy of the pDC lineage (Chaperot et al., 2001). By performing an immunophenotypic study on 7 BPDCN patients, they demonstrated that these tumor cells presented a distinct immunophenotype; lin⁻, CD123⁺, CD4⁺, CD56⁺, CD3⁻, CD13⁻, CD33⁻, and CD19⁻. More importantly leukemic cells upon IL-3 treatment, BPDCN cells became powerful inducers of T-cell proliferation and produced considerable amounts of IFN- α in response to inactivated influenza virus (Chaperot et al., 2001). Based on this and clinical and pathological criteria, BPDCN is classified within Acute Myeloid Leukemia (AML) and myeloid related neoplasms in the 2008 WHO classification of tumors (Fachetti, 2008).

Currently clinical strategies for BPDCN are restricted to conventional AML/ALL or lymphoma type chemotherapy. Relapses are frequent and invariably fatal, indicating the necessity for rationalized new therapeutic strategies for this disease. To date neither genetic nor clonal origin of this disease is known. Patients display complex chromosomal abnormalities affecting in decreasing frequency 5q (5q21 and 5q34), 12p (12p13), 13q (13q13 and 13q21), 6q (6q23-ter), 15q and chromosome 9 (Jardin et al., 2009, Leroux et al., 2002, Petrella et al., 2005). More recently, additional recurrent deletions of chromosomal regions have been identified such as (4q34, 9p13-p11, 9q12-q34 and 13q12-q31) (Dijkman et al., 2007). A single translocation t(2;17;8)(p23;q23;p23)

case has also been reported lately (Tokuda et al., 2014) and gene mutations affecting *TP53* (p53), *TET2* and *ASXL1* have been identified by next generation sequencing approaches (Jardin et al., 2009, 2011, Menezes et al., 2014).

In order to have a better understanding of molecular mechanisms underlying BPDCN oncogenesis, we have performed molecular and functional analysis of a novel, balanced chromosome translocation t(3;5)(q21;q31) observed in a BPDCN patient at our center (BPDCN cell line, GEN2.2) (Chaperot et al., 2006, Leroux et al., 2002). Our results demonstrated that this translocation targets the *NR3C1* gene encoding the glucocorticoid receptor (GCR), (located at 5q31), and a novel nuclear long non-coding RNA gene on 3q21, which here is named *lincRNA-3q*.

Chromosome 5q deletions are common in AML and myelodysplastic syndromes (MDS), pointing toward the pathogenic role of this region in these diseases (Jerez et al., 2012). Using both clinical data and biological samples from a large cohort of 47 BPDCN patients, gathered through the “Groupe Francophone Cytogénétique Hématologiques” and external collaborations (Besancon, France and Leiden, Netherlands), we demonstrate that *NR3C1* gene deletion is a critical pathogenetic event in BPDCN pathogenesis, conferring particularly dismal prognosis. *NR3C1* gene alterations have been already described in a subset of relapsing B-ALL, (Kuster et al., 2011, Mullighan et al., 2008).

Functional studies revealed the t(3;5) to encode a fusion protein, GCR-FP, which interfere with GCR signaling pathway including response to therapy. Strikingly, altered GCR signaling was also functionally linked to misregulation of major epigenetic signaling networks in particular polycomb-mediated gene expression repression.

In the second part of this work, we identified a novel nuclear long intervening non-coding RNA (lincRNA), called here *lincRNA-3q*. 3q rearrangements are known to confer extremely poor prognosis, at least in AML and MDS (Byrd et al., 2002, Grimwade et al., 2010, 2001, Jerez et al., 2012). Given, the emerging role of lincRNAs and the growing evidence of their implication in cancer (Gupta et al., 2010, Prensner et al., 2013, Tokuda et al., 2014, Yap et al., 2010), we investigated whether this RNA could be involved in disease progression, proposing a regulatory function for epigenetic signaling

pathways in BPDCN pathogenesis. We demonstrated marked deregulation of *lincRNA-3q* in high risk AML and BPDCN cell lines and primary patient samples. Our work provides evidence of a key role for *lincRNA-3q* in cell cycle progression at the G1/S transition as well as in the control of hematopoietic and leukemic stem cell signatures in myeloid leukemia. Finally we showed that epigenetic regulatory factors and BET inhibitors could suppress *lincRNA-3q*, providing therapeutic opportunities in BPDCN.

In summary, this work has led us to the characterization of two important target genes in leukemogenesis of BPDCN and potentially other myeloid and lymphoid neoplasms sharing similar biological features with this disease. By using functional genomic analysis we have identified deviant GCR signaling, and unscheduled activation of a novel nuclear lincRNA, driving deregulation of chromatin and transcription factor networks in these leukemic cells. And finally we propose BET bromodomain inhibition as a therapeutic strategy in BPDCN.

Results

NR3C1 deletion and a novel, BET inhibitor-sensitive, lincRNA drive disease pathogenesis in plasmacytoid dendritic cell neoplasms.

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ABSTRACT

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is an incurable malignancy for which disease mechanisms are unknown. Here, we identify the *NR3C1* gene (5q31), encoding the glucocorticoid receptor (GCR), and a long, intergenic, non-coding RNA gene (named here *lincRNA-3q*), respectively, as targets for genetic alteration or transcriptional deregulation in BPDCN. *NR3C1* translocation/deletion was associated to critically short survival in BPDCN and to abnormal activity of GCR, EZH2, and FOXP3 gene regulatory networks. *LincRNA-3q*, was found to encode a nuclear, non-coding RNA that is ectopically activated in BPDCN and high-risk AML. Depletion of *lincRNA-3q* in myeloid cancer cells induced cell cycle arrest, coincident to suppression of E2F1/Rb and leukemia stem cell-specific gene expression signatures. BET bromodomain protein inhibition could selectively suppress *lincRNA-3q* indicating a treatment strategy for counteracting oncogenic activity of this non-coding RNA. Thus, this work defines a new framework for understanding disease pathogenesis and treatment resistance in BPDCN.

INTRODUCTION

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare and clinically aggressive disorder that shows dismal prognosis whatever the treatment (Fachetti, 2008). Median overall survival is less than 2 years even with high dose chemotherapy, although longer term, albeit short-lived remissions have been observed in allo-transplanted patients (Feuillard et al., 2002; Ramanathan et al., 2013; Roos-Weil et al., 2013).

BPDCN is currently classified in the "acute myeloid leukemia (AML) and related precursor neoplasms" in the 2008 WHO classification (Swerdlow, 2008). However, the identity of the BPDCN cell of origin remains a matter of investigation (Chaperot et al., 2001; Osaki et al., 2013) and the precise pathogenic mechanisms underlying BPDCN are not currently known. Indeed, BPDCN presents unusual cytogenetic and molecular features, including co-occurrence of chromosomal aberrations typical of both myeloid and lymphoid disorders (Jardin et al., 2009; Leroux et al., 2002), a mutational landscape that is more typical of AML disease (Alayed et al., 2013; Jardin et al., 2011; Menezes et al., 2013), while showing predominant expression of lymphoid and pDC-restricted genes (Dijkman et al., 2007). Moreover, normal plasmacytoid dendritic cell development relies on the

stepwise, and cell context-specific activity of transcription factor and signalling receptor networks, that are dually implicated in either lymphoid or myeloid lineage development (Belz and Nutt, 2012).

Evolution from pre-existing myelodysplastic syndrome (MDS) or myeloproliferative disorders (MPD) has been described in a subset of BPDCN cases (Fachetti, 2008; Pagano et al., 2013). Deletion of chromosome 5q, commonly seen in MDS and AML, is frequent in BPDCN, albeit in association with 'lymphoid type' anomalies (up to 70% of cases with abnormal karyotypes) (Jardin et al., 2009; Leroux et al., 2002). This has led to speculation that initiating oncogenic events in BPDCN may target an early hematopoietic precursor (Jardin et al., 2009; Leroux et al., 2002). 5q deletion (outside of the 5q- syndrome) is associated with increased risk of leukemic transformation in MDS (Jerez et al., 2012) and to very poor prognosis in AML (Byrd et al., 2002; Grimwade et al., 2001). This is attributed to haploinsufficiency for critical 5q gene(s), that in cooperation with additional signalling networks, drives malignant transformation and clonal evolution in these disorders (Ebert, 2011). Similar 5q gene haploinsufficiency mechanisms in are likely to operate in BPDCN since recent sequencing efforts, although performed in only a few patients, have not revealed mutations in genes localising to 5q (Menezes et al., 2013). As in myeloid and lymphoid malignancies, mutations in key epigenetic modifier-encoding genes have been described in a proportion of BPDCN cases, thus supporting a role for deregulation of epigenetic signalling pathways in BPDCN pathogenesis (Alayed et al., 2013; Jardin et al., 2011; Menezes et al., 2013).

In an effort to gain insights into the molecular pathogenesis of BPDCN, we have performed molecular and functional analysis of a novel, balanced t(3;5)(q21;q31) observed in a BPDCN case recruited at our centre. We identify *NR3C1* as a critical, pathologically relevant 5q target gene in BPDCN. Additionally, we extend previous observations identifying a potential role for epigenetic modifier gene mutations in BPDCN pathogenesis by providing the first evidence of a key role for nuclear lincRNA deregulation in the pathogenesis of this disorder.

RESULTS AND DISCUSSION

5q anomalies in BPDCN confer adverse clinical outcome.

As a first step to uncovering new disease mechanisms in BPDCN, we screened 47 BPDCN patients at diagnosis for the presence of 5q alterations/chromosome 5 loss, by conventional metaphase karyotyping (n=39), aCGH (n= 25) and/or locus specific FISH (n=22). Overall, 5q alterations, including

a single case of balanced 5q translocation [(UPN1; t(3;5)(q21;q31) ; Fig. S1A] and one case with chromosome 5 loss, were observed in 17/47 patients (Fig. 1A). In cases with available cytogenetics (n = 39), with the exception of one case (UPN33), 5q alterations consistently occurred in the context of complex karyotypes in either founder clones (13 cases) or diagnostic subclones (3 cases) (Table S1). In a majority of cases, 5q loss spanned AML/MDS 5q deletion regions, in agreement with our previous findings (Jardin et al., 2009; Leroux et al., 2002). Consistent with a role in leukemia initiation and / or clonal evolution and disease progression, and as seen in myeloid malignancies, the presence of 5q anomalies in BPDCN was associated to extremely poor prognosis (p=0.001) (Fig. 1B, top panel).

Targeting of *NR3C1* by 5q alterations in BPDCN.

As an entry point to identification of pathogenetically relevant 5q genes in BPDCN, we focussed our attention on molecular cytogenetic characterisation of the balanced t(3;5)(q21;q32), identified in UPN 1 (BPDCN cell line GEN2.2) (Table S1, Fig. S1A) (Leroux et al., 2002). Involvement of chromosomal arm 3q, was of added interest as 3q aberrations are known to confer extremely poor prognosis, at least in AML and MDS (Byrd et al., 2002; Grimwade et al., 2010; Grimwade et al., 2001; Jerez et al., 2012). Remarkably, FISH mapping in GEN2.2 cells revealed the t(3;5) to target the *NR3C1* gene, located in 5q31 (Fig. 1C). *NR3C1* encodes the glucocorticoid receptor (GCR), a ligand dependent transcription factor of the nuclear hormone receptor family (Nicolaidis et al., 2010). More detailed analysis by aCGH and/or FISH analysis with an *NR3C1*-specific probe, in our cohort of BPDCN, showed *NR3C1* deletion to occur in 13 of 17 5q-rearranged cases (76%) of which 3 in subclones (Fig. 1A, Table S1). No further *NR3C1* translocations were identified. Strikingly, one case with normal cytogenetics (UPN33) presented a focal, morphologically cryptic, *NR3C1* deletion (coordinates 5:141,529,340 to 143,696,321), thus defining a minimally deleted 5q region in our BPDCN patient series. Focal deletions of *NR3C1* have previously been implicated in B cell acute lymphoblastic leukemia (Kuster et al., 2011; Mullighan et al., 2008) but not in myeloid malignancies. *NR3C1* deletions were mono allelic and sequencing, when performed (UPN 1 to 9), did not detect *NR3C1* mutations, suggesting that haploinsufficiency for *NR3C1* is at least one pathogenetically relevant event in 5q/*NR3C1* deletion/alteration in BPDCN. In keeping with this, *NR3C1*-deleted BPDCN showed lower *NR3C1* expression compared to non-(5q)-deleted cases, as assessed by gene expression profiling in primary BPDCN bone marrow or skin samples (Fig. 1D). *NR3C1* deletion was associated to

critically short survival, compared to BPDCN cases with 5q alterations that did not target *NR3C1* (Fig. 1B, lower panel). Taken together, our findings point to the existence of common pathogenetic mechanisms, centering on *NR3C1*, in BPDCN and B-ALL and suggest that screening for *NR3C1* alterations could serve to identify very high risk BPDCN patients.

Molecular characterisation of a novel t(3;5) in BPDCN reveals fusion of the *NR3C1* gene to a lincRNA gene in 3q21.

To further explore the pathological relevance of *NR3C1* rearrangement in BPDCN, we performed detailed molecular and functional characterisation of the t(3;5) observed in UPN1 (Fig. 2). RACE-PCR and sequencing revealed the t(3;5) to encode a single chimeric transcript comprising exons 1 and 2 of the *NR3C1* gene fused to an antisense sequence deriving from a novel *lincRNA* gene located in 3q21 (named here, *lincRNA-3q*) (Fig. 2A, Fig S1 and S5). LD-PCR genomic breakpoint cloning identified this transcript to result from fusion of *NR3C1* intron 2 (chr5:142,732,115) to *lincRNA-3q* intron 3 on the der(3) chromosome (chr3:129,837,689) (Fig. S1D). The *NR3C1-lincRNA-3q* fusion transcript thus likely derives from a splicing event between *NR3C1* exon 2 and a cryptic splice acceptor site located in the antisense orientation in *lincRNA-3q* exon 2 (chr3:129,832,478) (Fig. 2A, Fig. S1C). No further abnormal transcripts were detectable by Northern blotting across the *NR3C1* gene, thus ruling out *NR3C1* reciprocal fusion transcripts deriving from the der(5) chromosome (Fig. S1B). The *NR3C1-lincRNA-3q* fusion transcript was detected only in the t(3;5)-positive GEN2.2 cells and not in the t(3;5)-negative cell lines (Fig. S1B and C). Sequencing predicted the *NR3C1-lincRNA-3q* fusion transcript to encode a truncated GCR fusion protein (GCR-FP) comprising the GCR N-terminal activation domain, AF1 (amino acids 1 to 393), fused to 18 amino acids derived from transcriptional read through into the *lincRNA-3q* locus (Fig. 2B, left panel). In keeping with this, western blotting in t(3;5)-positive cells, using an anti-N terminal GCR antibody, detected a single abnormal GCR protein isoform (approximately 52kD, hereafter referred to as GCR-FP), in addition to the wildtype GCR (90kD) (Fig. 2B, right panel).

The t(3;5)-encoded GCR fusion protein drives altered GCR signalling and drug resistance in BPDCN.

NR3C1 gene alterations have been described in a subset of relapsing B-ALL, suggesting a role in therapy resistance (Kuster et al., 2011; Mullighan et al., 2008). We thus investigated responses to drug therapy following stable overexpression of a GFP-tagged isoform of GCR-FP in CAL-1 BPDCN cells, compared to CAL-1 cells expressing GFP alone (Fig. 2C, Fig. S2A-B). Strikingly, responses to combination therapy with high dose dexamethasone (10 μ M) and etoposide (10 μ M) were markedly reduced in CAL-1 GCR-FP-overexpressing cells, compared to controls (Fig. 2C). Cell death following dexamethasone (10 μ M) or etoposide (10 μ M) treatment alone was unchanged, at least under these conditions. We next asked whether GCR-FP overexpression might dominantly interfere or result in functional haploinsufficiency for wild type GCR alpha transcriptional transactivation activity. To test this, GCR activity luciferase reporter assays were performed by transient co-transfection assays in Cos cells expressing wildtype GCR alpha and increasing amounts of GCR-FP, in the presence or absence (DMSO) of dexamethasone, a synthetic GCR ligand (Fig. 3A ; 6h, 100nM). This revealed dose-dependent inhibition of GCR transcriptional transactivation activity indicative of functional interference. We next investigated the impact of the t(3;5)-encoded GCR-FP on global, ligand-dependent GCR signalling. For this, as a model system, we used CAL-1 GCR-FP-overexpressing cells, treated for 6 hours with low dose dexamethasone (100 nM) or vehicle (DMSO), (Fig. 3B, C). Low as opposed to high dose dexamethasone was used in order to simulate the conditions in which *NR3C1* alterations might be expected to undergo clonal selection in treatment naïve leukemia cells *in vivo* (i.e. physiological glucocorticoid concentrations). Strikingly, this analysis revealed specific, hormone-dependent deregulation, by GCR-FP, of a discrete subset of genes (83 genes; 74 up and 9 down), including known GCR target genes such as *C-FOS*, and genes encoding nuclear factors that have been implicated in either normal or leukemic lymphoid (*BCL6*) (Bunting and Melnick, 2013), myeloid (*c-MYB*) (Clappier et al., 2007; Lahortiga et al., 2007) or dendritic cell lineage development (*RELB*) (Belz and Nutt, 2012) (Fig. 3B).

Deregulation of GCR, EZH2 and FOXP3 gene regulatory circuits is a hallmark of 5q alterations targeting *NR3C1* in BPDCN.

To further explore the pathological relevance 5q alterations targeting *NR3C1* in BPDCN, we used GSEA to identify, in an unbiased manner, dexamethasone-dependent gene signatures that are abnormally modulated upon overexpression of the t(3;5)-encoded GCR-FP in BPDCN cells. Consistent with our functional studies (Fig. 2C), this analysis revealed enrichment of a prednisone resistance gene signature previously described in B-ALL (Holleman et al., 2004), in dexamethasone-treated, CAL-1-GCR-FP-expressing cells, compared to control-treated cells (Fig. 2D), in addition to enrichment for signatures related to EZH2 loss of function in cancer cells (Kondo et al., 2008; Takeda et al., 2006) (Fig. 3C and Fig. S3A). Additionally, a significant proportion of the genes that were abnormally modulated upon hormone treatment in CAL-1-GCR-FP-expressing cells, compared to controls, was found to correspond to FOXP3-bound genes, as identified by ChIP-seq studies in CD4-positive T helper cells (Zheng et al., 2007) (Fig. 3C). These genes included among others, a number that encode chromatin modifiers or nuclear factors such as BCL11B which is essential for early T cell lineage commitment, specifically for suppression of alternate myeloid or NK cell fates (Ikawa et al., 2010) and that is implicated in T cell malignancies (Gutierrez et al., 2011). Interestingly, FOXP3-bound genes that are upregulated in developing CD4 positive T lymphocytes in the thymus, including the *NR3C1* gene itself, appeared to be specifically affected upon hormone treatment of our model GCR-FP-overexpressing BPDCN cell line, compared to control treated cells, suggestive of compromised FOXP3 repressor activity (Fig. 3C). In view of this, we hypothesised that BPDCN with *NR3C1* deletion and thus possible haploinsufficiency for GCR function, might display similar deregulation phenotypes. To test this, gene expression profiling (GEP) was performed in RNA derived from the bone marrow of 11 BPDCN cases (3 with and 9 without 5q/*NR3C1* deletion). Additionally we reanalysed data from the skin biopsies of 5 BPDCN patients (2 with and 3 without 5q/*NR3C1* deletion) (Dijkman et al., 2007). In keeping with our starting hypothesis GSEA of these GEP data sets, revealed specific enrichment of the EZH2 loss of function and FOXP3 regulatory signatures in the 5q/*NR3C1* deleted versus non-deleted cases, thus arguing in favour of the pathological relevance of functional deregulation of these pathways in 5q/*NR3C1*-deleted BPDCN (Fig. 3C and Fig. S3B). To investigate this further we focused on EZH2, a member of the PRC2 repressor complex, that is a frequent target for deregulation in myeloid cancers, including BPDCN (Menezes et al., 2013; Shih et al., 2012) and that is required for

FOXP3 transcriptional repressor functions, at least in regulatory T cells (Arvey et al., 2014). Also, FOXP3 transcripts, albeit at very low levels, are detectable in normal, sorted human pDC cells and in bone marrow and skin biopsies from BPDCN patients (Fig. S4 ; expression analysis from GEO data sets GSE28490 (Allantaz et al., 2012) and GSE32719 (Pang et al., 2011) and data obtained or reanalysed in this study. We selected the CAL-1 GCR-FP-overexpressing cell line as a suitable model system to assess the impact of altered GCR signalling on global H3K27me3 levels (Fig. 3). Strikingly, this revealed evidence of a global decrease in H3K27me3 levels in CAL-1 GCR-FP-overexpressing cells treated with low dose dexamethasone (100nM or 500nM for 6 or 24h), compared to control treated cells (Fig. 3D, Fig. S2C). Coincident to this, reduced H3K27me3 levels were also observed at the *HOXA* locus, a major downstream target of EZH2 in the hematopoietic lineage, as assessed by site specific ChIP at the *HOXA5*, *HOXA9* and *HOXA11B* gene promoters (Fig. 3E). Taken together, these data indicate that altered GCR signalling thresholds, in addition to driving treatment resistance, can also directly contribute to leukemogenesis and clonal evolution, through interference with gene regulatory circuits requiring, at least in BPDCN, EZH2 and/or FOXP3 activity. Functional interference by GCR ('transrepression') of the activity of numerous transcription factors, by either direct binding or through chromatin dependent mechanisms is well documented (John et al., 2011; Nixon et al., 2013). Our findings raise the possibility that this facet of GCR function may play a role in leukemia.

***LincRNA-3q* is overexpressed in BPDCN and AML and encodes a nuclear non-coding RNA that regulates cell proliferation and leukemic stem cell gene expression signatures.**

We next wished to assess the pathological relevance of the lincRNA gene target identified at the 3q21 breakpoint in the t(3;5) observed BPDCN. For this, we first measured expression levels of *lincRNA-3q* in normal pDC, BPDCN cell lines and primary BPDCN leukemia samples by RT-qPCR (Fig. 1E). This revealed evidence of illegitimate *lincRNA-3q* activation in 7/10 primary BPDCN cases, compared to normal pDC (Fig. 1E). Evidence of *lincRNA-3q* deregulation was also evident in AML with intermediate or poor risk cytogenetics, particularly those AML cases with 3q abnormalities [inv/translocation (3q)] (Fig. 1E). This is of interest, since the *lincRNA-3q* locus is located approximately 1Mb telomeric of the inv/translocation 3q super enhancer region/*RPN1* recently identified as responsible for long range deregulation of the *EVI1* gene in AML (Groschel et al., 2014). *LincRNA-3q* overexpression appeared to occur independently of 3q genetic aberrations in BPDCN. Taken together these findings evoked a

potentially key pathological role for *lincRNA-3q* overexpression in the pathogenesis of BPDCN and AML. Additional RT-qPCR screening revealed broad expression across a wide range of normal tissues, with highest expression being observed in bone marrow, brain and kidney (Fig. S6A). Further screening hinted at deregulation in lymphoid and solid cancers as well, particularly in lymphoma, lung and liver cancer lines (Fig. S6B and C). Linc-RNAs can be either nuclear where they can operate as epigenetic regulators through RNA-dependent recruitment of chromatin modifier complexes to chromatin or cytoplasmic where they can operate as miRNA decoys (Ulitsky and Bartel, 2013). We thus assessed the subcellular localisation of *lincRNA-3q* by cell fractionation analysis in CAL-1, GEN2.2 and U937 AML cells, followed by RT-qPCR analysis of RNA extracted from whole cells, and cytoplasmic and nuclear fractions (Fig. 4A). Strikingly, *lincRNA-3q* was found to be predominantly nuclear (Fig. 4A), suggestive of a nuclear function, most probably related to chromatin signalling. To probe this further, we performed short hairpin RNA knockdown by using U937 as model cell line (overexpresses *lincRNA-3q* and is amenable to lentiviral transduction). Quite strikingly, *lincRNA-3q* knockdown (Fig. 4B) induced a G1/S arrest in these cells (Fig. 4C) without inducing cell death or evidence of differentiation at least at the morphological level (Fig. S7A and data not shown). In view of this phenotype, gene expression profiling, followed by GSEA, was performed in *lincRNA-3q* knockdown compared to control U937 cells, transduced with a non-targeting hairpin. Short hairpin-mediated *lincRNA-3q* depletion was associated to differential expression of 904 genes of which 428 were up and 476 down-regulated, thus suggestive of a role in both gene activation and repression. Of these, were multiple leukemia-relevant genes including *MYC*, *EZH2* and genes encoding E2F family members (E2F7 and E2F8) known to negatively regulate transcriptional activatory E2F members (Chen et al., 2009). Consistent with this and our data showing G1/S arrest in *lincRNA-3q*-depleted leukemia cells, GSEA revealed reduced expression of numerous E2F target genes upon *lincRNA-3q* depletion in U937 cells compared to controls (Fig. 4D). Additionally, knockdown of *lincRNA-3q* in U937 AML cells was also found to downregulate normal and leukemic hematopoietic stem cell gene expression programmes (Georgantas et al., 2004; Wang et al., 2010), compared to controls (Fig. 4D) suggestive of a role in the hematopoietic stem cell compartment which is consistent with high level *lincRNA-3q* expression in normal bone marrow samples (Fig. 1E). Thus, *lincRNA-3q* displays features of a bona fide leukemic driver that warrants further functional analysis and assessment for therapeutic intervention.

Numerous cancer genes have been shown to critically depend for their expression on the activity of bromodomain proteins of the Bromo and Extra Terminal (BET) domain family (Emadali et al., 2013; Groschel et al., 2014; Loven et al., 2013). Since we suspected epigenetic mechanisms to be the root cause of *lincRNA-3q* deregulation in leukemia cells we tested the effects of one such BET inhibitor (JQ1) on expression levels of *lincRNA-3q* in BPDCN and AML cell lines (CAL-1 and U937, respectively). Quite strikingly, *lincRNA-3q* levels were significantly suppressed in a dose and time-dependent manner in CAL-1 BPDCN cells and U937 cells (Fig. 4E) in parallel to MYC suppression (Fig. S7B). Thus BET bromodomain inhibition would be expected to counteract at least some of the oncogenic signalling properties of *lincRNA-3q* in BPDCN and AML.

In conclusion, our personalised, functional genomics analysis has identified deviant GCR signalling, and unscheduled activation of a novel nuclear *lincRNA*, to drive deregulation of chromatin and transcription factor networks of relevance to treatment resistance and malignant reprogramming in the dendritic cell lineage. Further studies will be required to fully leverage these findings for treatment innovation in BPDCN and potentially other myeloid and lymphoid cancers that share biological features with this disease. One avenue of investigation is BET protein inhibition.

MATERIALS AND METHODS

BPDCN patients investigated in this study were selected from 2 previously published French cohorts (Jardin et al., 2009; Leroux et al., 2002), 1 Dutch study (Dijkman et al., 2007) and via collaborations with the GFCH (Groupe Francophone de Cytogénétique Hématologique) and the French BPDCN network (Table S1 ; n= 47; median age: 66y, range: 7-82). Two human BPDCN cell lines GEN2.2 and CAL-1 derived from primary human BPDCN cells (Chaperot et al., 2006; Maeda et al., 2005) were included in the study and are referred to as UPN 1 and 2, respectively, in Table S1. Cell lines, were cultured in RPMI medium supplemented with 10% FCS (Chaperot et al., 2006; Maeda et al., 2005). Murine stromal cell support (MS5) was provided for GEN2.2 cells, as described (Chaperot et al., 2006). R-banded karyotyping, FISH analyses and array CGH were performed by standard methods, as previously described (Fournier et al., 2010; Jardin et al., 2009). BAC and fosmid probes used for FISH are listed in Table S2. Long distance (LD)-PCR cloning of the t(3;5) genomic breakpoint was performed using the Roche LD Expand kit and LD-PCR primers (Table S3). For RACE-PCR, RT-qPCR, northern blotting and Affymetrix gene expression analysis, total RNA was prepared by Trizol

reagent, according to the manufacturer's instructions and processed as described (Emadali et al., 2013). RACE-PCR (5' and 3') was used to clone the t(3;5)-encoded fusion transcript and was performed using BD Biosciences Clontech SMART RACE cDNA amplification kit, according to the manufacturer's instructions. RACE primers, are listed in Table S4. For functional studies, GCR fusion-protein cDNA was cloned into pcDNA3.1 or pEGP-C1 or as a GFP-tagged isoform into a modified pHIV-SFFV-mRFP-WPRE lentiviral vector, using primers described in Table S5. CAL-1 cell lines stably expressing GFP alone or GFP-tagged GCR fusion protein were obtained by lentiviral transduction and cell sorting (FACS ARIA, BD). Successful transduction was confirmed by flow cytometry and western blotting using anti N- and C-terminal GCR (respectively clones E-20 and P-20, Santa Cruz) and anti-GFP (clone B2, Santa Cruz) antibodies. Transient transfection assays, using the glucocorticoid response element Luciferase reporter plasmid (gift from J. Cidlowski) were performed with lipofectamine reagent in COS-7 cells. For gene expression profiling, control and GFP-tagged, GCR-FP-expressing, CAL-1 cell lines were treated with vehicle (DMSO) or dexamethasone (100nM) for 6h and total RNA extracted for hybridization to Affymetrix U133 2.0 chips, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). For *lincRNA-3q* knockdown, short hairpin targeting sequences were cloned into pLKO and packaged for lentiviral transduction into CAL-1 cells. Selection was by puromycin for 10 days. Cell cycle analysis and Affymetrix gene expression profiling were performed as described (Emadali et al., 2013). For detailed experimental methods and description of bioinformatics analyses, see supplemental methods.

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FIGURE LEGENDS**Figure 1: Targeting of the *NR3C1* gene by 5q deletions/translocation in BPDCN.**

A. Schematic representation of 5q alteration in BPDCN patients (n= 17), as indicated. **B.** Kaplan-Meier cumulative survival curves in BPDCN according to 5q alteration status (for simplicity this is labelled del(5q) [UPN1, 2, 8,12, 14, 21, 22, 24, 26, 27, 29, 31, 35 and 45] and non-del(5q) [UPN3, 9, 10, 16-19, 25, 28, 36, 37, 40-42] (upper panel) and *NR3C1* deletion status [UPN1, 8, 14, 22, 24, 26, 27, 31, 35 and 45] (lower panel ; determined by FISH or aCGH; $\Delta NR3C1$), p values from log-rank test. **C.** FISH analysis of the t(3;5)(q21;q35) translocation in the BPDCN cell line, GEN2.2 with chromosome 5q probes CTB-88J14 (green) and RP11- 278J6 (red) (Table S2). Note a split signal for RP11-278J6 (*NR3C1*) on the der(3) and der(5) chromosomes. **D.** Affymetrix-derived *NR3C1* expression in BPDCN patients presenting del(5q) targeting *NR3C1* or not. **E.** RT-qPCR-derived *lincRNA-3q* expression profiles in normal bone marrow (BM), normal pDC, BPDCN (CAL-1, GEN2.2) and AML cell lines (U937) and in BPDCN and AML patient samples. AML patients are classified according to cytogenetic risk group (Fav: favorable, Interm: Intermediate, Adv: Averse). Cases presenting chromosome 3q abnormalities are identified in blue.

Figure 2: The t(3;5)-encoded GCR-lincRNA-3q fusion is associated with glucocorticoid resistance.

A. Schematic representation of the genomic organization surrounding the normal 3q and der(3) breakpoint region and the resulting fusion transcript *NR3C1-lincRNA-3q*. cen: centromere ; tel: telomere ; blue filled circle: GATA2 super enhancer ; genes as indicated ; TSS: transcription start site ; ex: exon. **B.** Schematic representation of the structure of wild-type GCR (left panel, top) and predicted GCR fusion protein (left panel, bottom). NTD: N-terminal domain, DBD: DNA binding domain, LBD: ligand binding domain, AF: activation function domain, ZnF: zinc finger domain, (right) GCR western blot analysis showing expression of an abnormal GCR isoform (GCR-FP) exclusively in GEN2.2 cells, compared to t(3;5)-negative cell lines, as indicated. **C.** Evaluation of drug sensitivity of CAL-1 cells overexpressing GCR-FP compared to control CAL-1 cells, following 16h treatment with 10 μ M etoposide (Eto), 72h treatment with 10 μ M dexamethasone (Dex) or a combination of both treatments. Specific cell death was calculated as follows: 100 - [experimental cell death (%) - spontaneous cell death (%)]/100% - spontaneous cell death (%). n=3, p value from a Wilcoxon test. **D.** Gene Set

Enrichment Analysis (GSEA) enrichment plots obtained using the ratio Dex/DMSO in CAL-1-GCR-FP (GCR-FP(+)) over the ratio Dex/DMSO in CAL-1 control cells (GCR-FP(-)).

Figure 3: Misregulated GCR and EZH2 signaling as a consequence of NR3C1 alterations in BPDCN.

A. Luciferase reporter assay for glucocorticoid transcriptional transactivation activity in Cos cells transduced with empty pcDNA3.1 vector, pcDNA3.1-GCR and pcDNA3.1-GCR-FP, as indicated, after a 6h treatment with either DMSO or 100 nM dexamethasone. **B.** Heat-map representation of the differentially expressed genes between CAL-1-GCR-FP [GCR-FP(+)] and CAL-1 control cells [GCR-FP(-)] treated with 100 nM dexamethasone for 6h. Black arrows: genes cited in text. **C.** Gene Set Enrichment Analysis (GSEA) enrichment plots showing gene regulatory circuits that are differentially expressed between dexamethasone treated CAL-1-GCR-FP compared to control CAL-1 control cells (CAL-1-GFP) and between BPDCN patients presenting or not del(5q) targeting *NR3C1* (bone marrow or skin, as indicated). **D.** Western blot analysis of global H3K27me3 in CAL-1-GCR-FP [GCR-FP(+), upper panel] and CAL-1 control cells [GCR-FP(-), lower panel] treated with 100 nM dexamethasone for 6h and 24h. **E.** ChIP for H3K27me3 followed by qPCR analysis for enrichment on *HOXA* gene promoters, as indicated, in CAL-1-GCR-FP [GCR-FP(+)] and CAL-1 control cells [GCR-FP(-)] treated with 100 nM dexamethasone for 24h.

Figure 4: *lincRNA-3q* overexpression in BPDCN and AML drives G1/S and leukemia stem cell gene expression signatures and can be suppressed BET inhibition.

A. RT-qPCR and RT-PCR analysis of *lincRNA-3q* and GAPDH (control) expression levels in CAL-1 BPDCN cells in RNA extracted from whole cell and subcellular fractions ; total, whole cell extract ; cyt : cytosolic fraction, nuc: nuclear fraction (n=2). **B.** RT-qPCR-derived *lincRNA-3q* expression in U937 cells transduced with control (Ctrl) or *lincRNA-3q* targeting sh constructs (shA, B and C). **C.** Cell cycle analysis of U937 transduced with control (Ctrl) or *lincRNA-3q* sh (shA, B and C) constructs, (upper panel) histogram representation of % cells in cell cycle phases, (lower panel) (n=4). **D.** Gene Set Enrichment Analysis (GSEA) enrichment plots obtained by comparing gene expression profiles of U937 cells transduced with control (shCtrl) or *lincRNA-3q* targeting sh (shlincRNA-3q) and associated

heat-map. Genes mentioned in the text are marked with an arrow. **E.** RT-qPCR-derived *lincRNA-3q* expression in U937 and CAL-1 cells treated with JQ1 at the time and doses indicated (n=3).

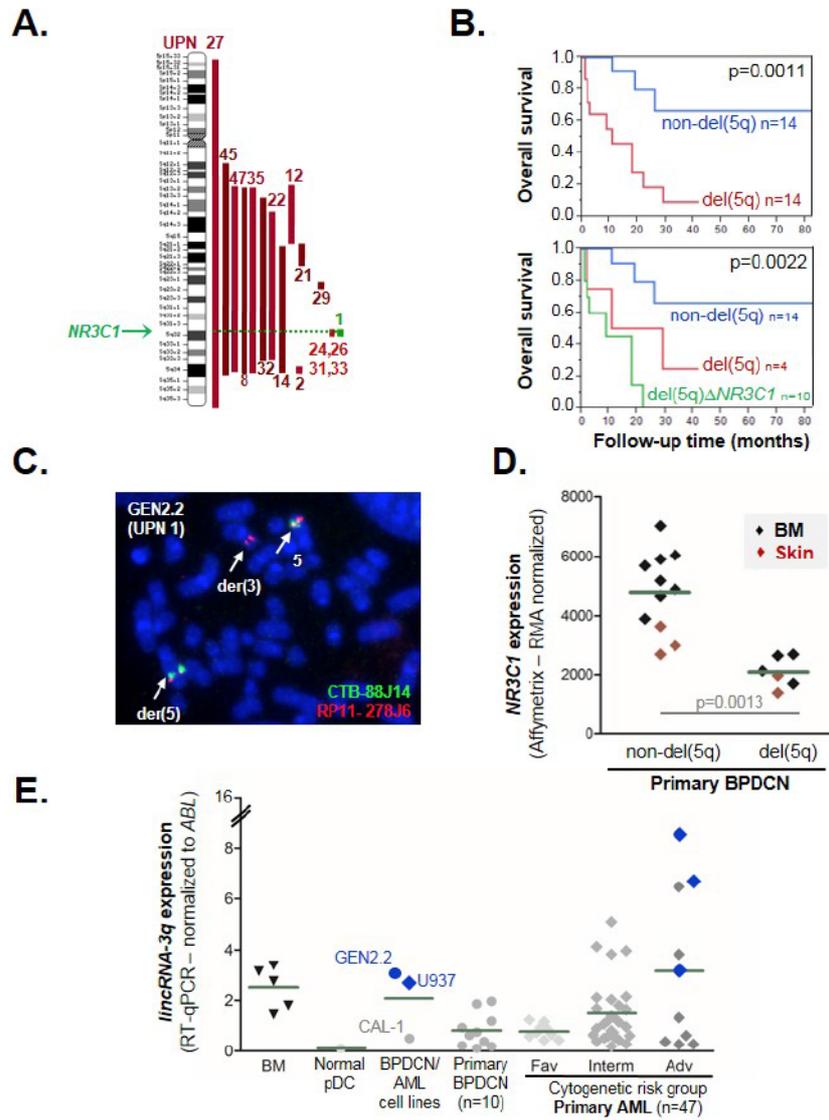


Figure 1

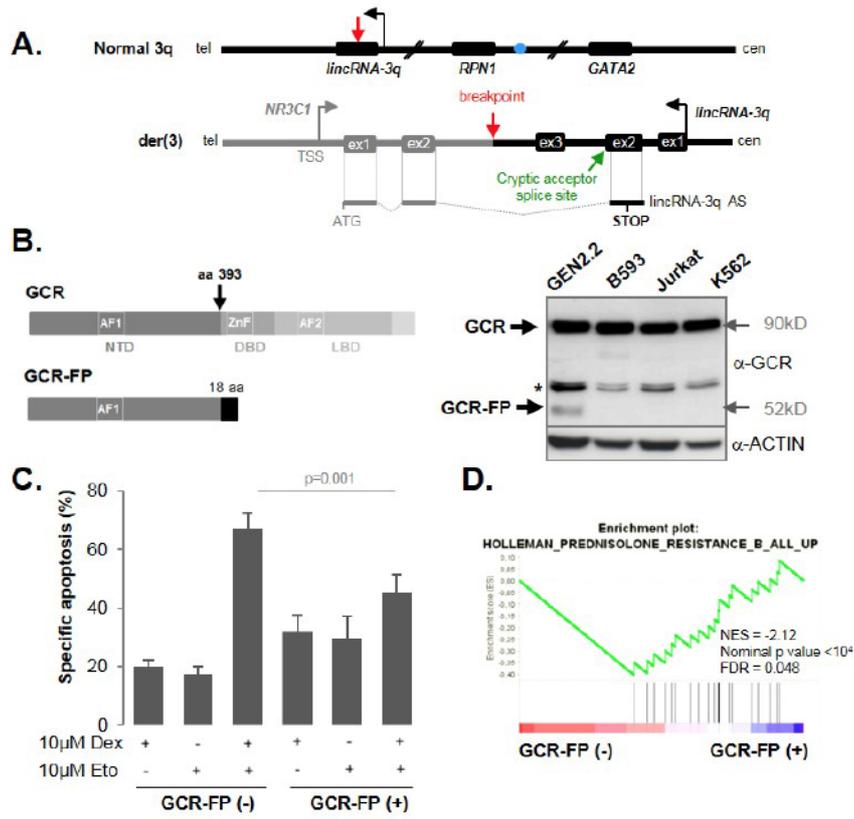


Figure 2

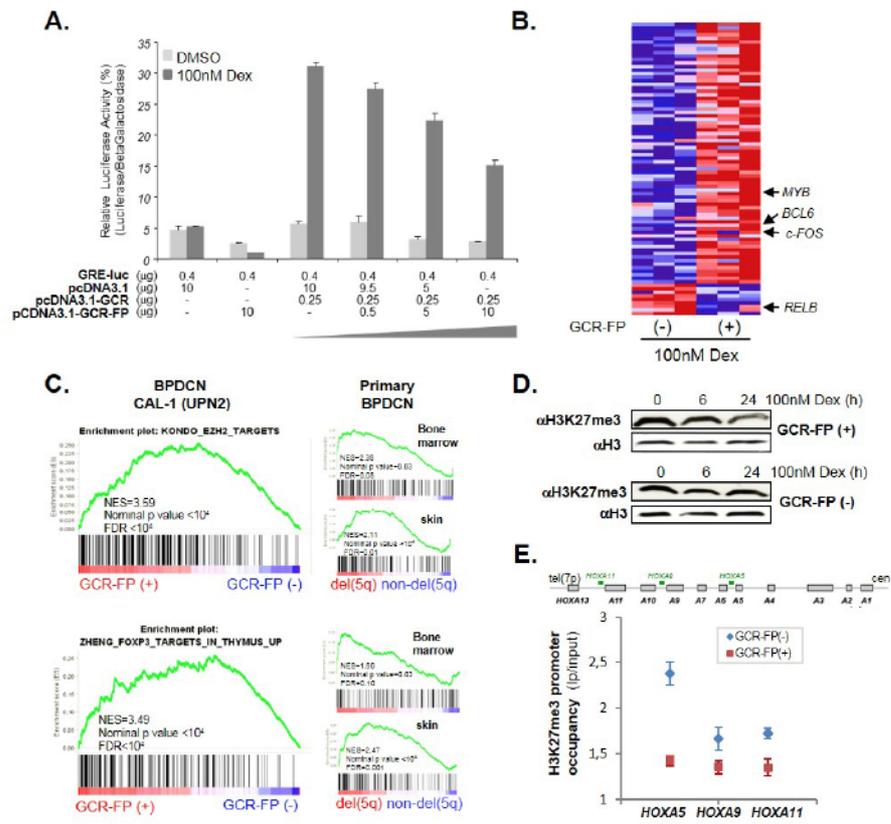


Figure 3

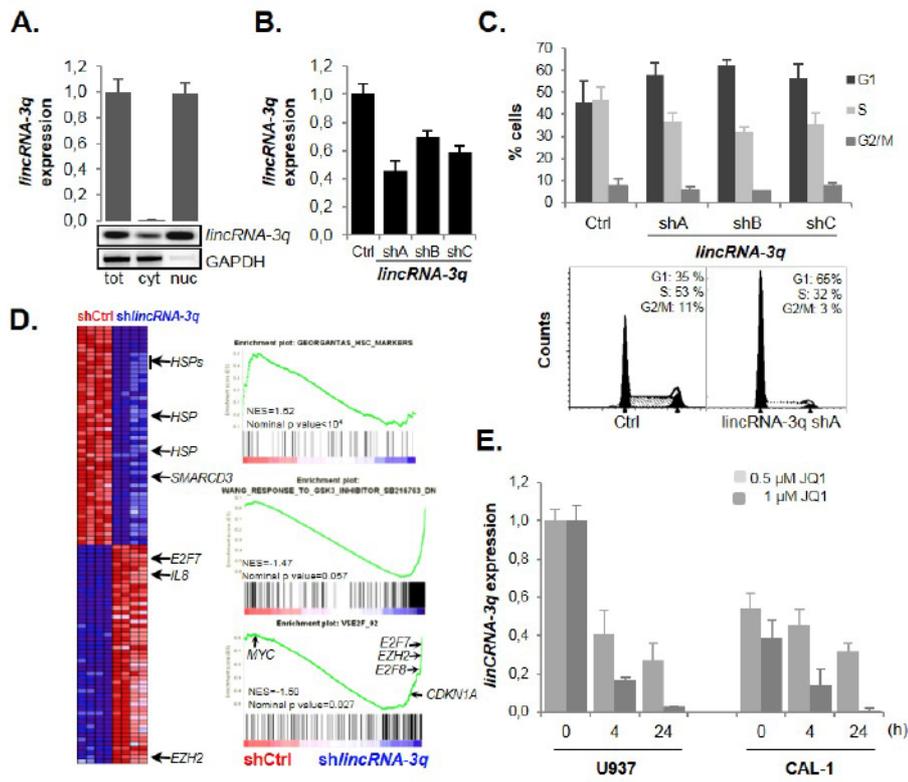


Figure 4

SUPPLEMENTAL METHODS

FISH

FISH analyses were performed by standard methods, as described (Fournier et al., 2010). BACs probes used for FISH are listed in Table S2 and were selected through the UCSC or ENSEMBL genome browsers.

Long distance (LD)-PCR

LD-PCR products were generated from 0.5 µg total DNA using the Expand Long Template PCR System (Roche) and the following conditions for amplification: initial denaturation 94°C, 2 min; 94°C, 10s – 57°C, 30s – 68°C, 20 min (x10); 94°C, 15s – 57°C, 30s - 68°C, 20 min + 20s per cycle (x24); final extension, 68°C, 7min. PCR products were then gel-purified, cloned into pGEM-T Easy vector (Promega) and sequenced in both directions. LD primers are listed in Table S3.

RNA extraction

Total RNA was extracted from cell lines, tissues or sorted B cells by TRIzol reagent (Invitrogen) and quantified by NanoDrop (Thermo Fisher Scientific).

RACE-PCR

Nested RACE PCR reactions were performed from 1 µg RNA using the SMART RACE cDNA Amplification kit (BD) with *NR3C1* exon 2 specific forward (3' RACE) and reverse primers (5' RACE) together with 5' and 3' RACE SMART primers, in order to identify *NR3C1* exon 2 flanking sequence (see Table S4), using the manufacturer's instructions. RACE products were purified on agarose gels, cloned and sequenced in both directions.

RT-qPCR gene expression analysis

Total RNA was reverse transcribed using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen), according to manufacturer's instructions. qPCR was performed using SYBR® Green PCR master mix (Applied Biosystems) and primers shown in Table S4, according to the manufacturer's instructions. The *ABL* gene was used as a control for normalization of gene expression data. qPCR was performed on a MX3000P machine (Stratagene).

Fusion transcript cloning

For functional studies, GCR fusion-protein cDNA was cloned into pcDNA3.1 and then subcloned in pEGP-C1. N-terminal GFP-tagged GCR fusion protein cDNA was then subcloned as a 2117bp fragment BamH1(ATG)/Xho-1(TGA) into the pHIV-SFFV-mRFP-WPRE vector using primers described in Table S5.

Northern Blotting

20 µg RNA was electrophoresed on 1% denaturing gels, transferred onto Hybond N+ membrane and hybridized using *NR3C1* cDNA probes obtained by RT-PCR amplification using primers listed in Table S6.

Lentiviral Transduction

N-terminal GFP-tagged GCR fusion protein cDNA was then subcloned as a BamH1(ATG)/Xho-1(TGA) 2117bp fragment into the pHIV-SFFV-mRFP-WPRE vector using primers described in Table S6. Non-targeting or *lincRNA-3q* targeting short hairpin (sh) RNA sequences were designed using the DSIR algorithm (<http://biodev.cea.fr/DSIR/DSIR.html>) (Vert et al., 2006). Sequences provided in Table S7, were cloned into the pLKO-1 lentiviral vector (Addgene). Lentiviral particles were packaged, as described previously (Levy et al., 2010). Cells were transduced with lentiviral particles at a MOI (multiplicity of infection) of 10. GFP expressing cells were sorted using flow cytometry (FACS ARIA, BD). Cell lines stably expressing shlincRNA-3q were established under puromycin selection.

Western Blotting

Protein lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for blotting with anti N- and C-terminal GCR (respectively clones E-20 and P-20, Santa Cruz), anti-GFP (clone B2, Santa Cruz), anti-H3K27me3 (Millipore), anti-H3 (Upstate) and anti-ACTIN (Sigma) antibodies. After treatment with blocking solution (1X PBS, 8% skimmed milk, 0.1% Tween 20), membranes were incubated with primary antibody (GCR, 0.4 µg/ml, H3K27me3, H3 and actin 0.5 µg/ml in PBS 3% skimmed milk 0.1% Tween 20), washed and incubated with anti-rabbit or mouse IgG-HRP (Thermo Fisher Scientific) before incubation in ECL SuperSignal West Pico

Chemiluminescent Substrate (Thermo Fisher Scientific). Images were captured using autoradiography films.

GRE reporter assay

Transient transfection assays using the glucocorticoid response element Luciferase reporter plasmid (gift from J Cidlowski) were performed in COS7 cells using Lipofectamine (Invitrogen) according to manufacturer's instructions.

ChIP on native chromatin

ChIP experiments were performed as described in (Fournier et al., 2010). Briefly, all Q-ChIP assays were performed in triplicate in at least two independent chromatin preparations. For qPCR analyses of immunoprecipitated chromatin fractions at sequences of interest, a SYBR Green PCR kit (Applied Biosystems) was used. qPCR was performed on 20 ng of ChIP DNA or DNA from input chromatin fractions, using the following conditions; 40 cycles at 95°C for 15 s, 60°C for 1 min. Primer pairs are listed in Table S8. qPCR data obtained on immunoprecipitated fractions were normalized to input chromatin ($IP/Input = 2^{\Delta Ct} = 2^{[Ct(IP)-Ct(Input)]}$). Background precipitation was evaluated with a mock IP.

Cell fractionation

Nuclear fractions were prepared by incubating cells in 0.2% NP40, 0.3M sucrose, 15mM Tris pH8, 60mM KCl, 15mM NaCl, 5mM MgCl₂, 0.1mM EDTA pH8 and protease inhibitors (Complete®, Roche) for 10 min on ice. Efficient cell lysis without nuclear disruption was verified by trypan blue staining prior to nuclear fraction separation by centrifugation at 500g, for 10 min at 4°C.

Affymetrix gene expression analysis

Affymetrix microarrays were processed in the Microarray Core Facility of the Institute of Research on Biotherapy, CHRU-INSERM-UM1 Montpellier (<http://irb.chu-montpellier.fr/>). RNA was amplified using the kit GeneChip IVT express and hybridized to HG U133 Plus 2.0 microarrays according to manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified using the GCOS 1.2 software. Gene expression data were normalized with RMA algorithm and analyzed using GeneSpring

software package (Agilent). Data are being deposited in the NCBI Gene Expression Omnibus (pending accession number).

GSEA analysis

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states (Subramanian et al., 2005). We first generated a pre-ranked list obtained by calculation of the ratio: fold change in CAL-1 cells transduced with GCR-FP upon dexamethasone treatment on fold change in control CAL-1 cells upon dexamethasone treatment. This strategy was defined to identify gene signatures specifically associated with the presence of GCR-FP upon dexamethasone treatment. Analysis was performed using GSEA v2.07 software to compare this pre-ranked list against the c2 gene sets from the Broad Institute Molecular Signature Database (<http://www.broadinstitute.org/gsea/msigdb/>). For shlincRNA-3q data analysis, GSEA was run using default parameters against the c2 and c3 gene sets from the Broad Institute Molecular Signature Database. Genes are ordered on the x axis by their rank in the list (each vertical line representing a gene). A detailed description of GSEA methodology and interpretation is provided online (<http://www.broadinstitute.org/gsea/doc/GSEAUUserGui-deFrame.html>). In brief, the normalized enrichment score (NES) provides “the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes”. The false discovery rate q -value (FDR q -val) is “the estimated probability that a gene set with a given NES represents a false positive finding”.

Statistics

Excel[®] 2010 (Microsoft), J.M.P.[®] 10.0.0 (SAS Institute) and GraphPad (Prism) softwares were used for graphical representations and to perform statistical analysis across all experiments. The Wilcoxon non-parametric method or the two-sided Student t tests were used for statistical analysis as indicated. Histograms represent the average for each group and errors bars represent standard deviation.

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SUPPLEMENTAL FIGURE LEGENDS**Figure S1: Molecular characterization of the t(3;5) translocation in the BPDCN line GEN2.2.**

A. Partial karyotype of the t(3;5)(q21;q31) from GEN2.2 cell line (UPN1). **B.** *NR3C1* northern blot analysis in a panel of leukemia and lymphoma cell lines, as indicated, showing expression of an abnormal GCR transcript (2 kb), exclusively in GEN2.2 cells using probe 1 (see Table S6: primers for *NR3C1* cDNA probe amplification). **C.** Sequence and schematic representation of the *NR3C1-lincRNA-3q* fusion transcript breakpoint junction (upper and middle panels), and RT-PCR fusion transcript detection in t(3;5)-negative CAL-1 cells compared to t(3;5)-positive GEN2.2 cells (lower panel, green arrow), using primers depicted in green (see also Table S4). **D.** Sequence and schematic representation of the der(3) genomic breakpoint (upper and middle panels). Long-distance (LD)-PCR amplification of the breakpoint junction in t(3;5)-positive GEN2.2 cells compared to control CAL-1 cells (lower panel), using primers depicted in blue (see also Table S3). A 16.2kb fragment can be amplified only in GEN2.2/UPN1 harbouring the t(3;5)(q21;q31) translocation. *non-specific band.

Figure S2: Stable expression of GFP-tagged GCR-FP in the BPDCN line CAL-1.

A. Western blot using both anti-GCR (upper panel) and anti-GFP (lower panel) antibodies in the parent CAL-1 cell line (WT) compared to CAL-1 cells transduced with empty GFP vector (n=2, GFP1 and GFP2) or with the GFP-tagged, GCR-FP (n=2, GCR-FP1 and GCR-FP2) expression vector, as indicated. **B.** Flow cytometric analysis of GFP and GFP-GCR-FP expressing CAL-1 cells after GFP flow cytometric cell sorting and cell line amplification *in vitro*. **C.** Western blot analysis of global H3K27me3 in CAL-1-GCR-FP [GCR-FP(+), upper panel] and CAL-1 control cells [GCR-FP(-), lower panel] treated with 500 nM dexamethasone for 6h and 24h.

Figure S3: Gene signatures abnormally induced by dexamethasone treatment of GCR-FP-expressing CAL-1 cells compared to control GFP-expressing cells.

A. Gene Set Enrichment Analysis (GSEA) enrichment plots obtained using the ratio Dex/DMSO in CAL-1-GCR-FP over the ratio Dex/DMSO in CAL-1 control cells. GSEA enrichment plots thus represent gene signatures specifically associated with the presence of GCR-FP upon dexamethasone treatment (left). **B.** Gene Set Enrichment Analysis (GSEA) enrichment plots obtained following

comparison of non-del(5q) and del(5q) primary BPDCN cells (source : skin biopsies or bone marrow – BM, as indicated).

Figure S4 FOXP3 transcript levels in human dendritic and CD4 T cell compared to BPDCN.

FOXP3 mRNA expression levels in hematopoietic stem cells (HSC, negative control), sorted, normal CD4 single positive thymic T cells, normal pDC and in BPDCN (patient bone marrow and skin samples and cell lines, this study), as indicated. Gene expression data for normal CD4 and pDC cells were derived from the GEO data set GSE28490. Data for normal HSC were derived from GEO data set GSE32719.

Figure S5: Genomic organisation of the *lincRNA-3q* locus.

A. Schematic representation of the *lincRNA-3q* genomic locus, showing lincRNA and TUCP transcripts from RNA-seq data, and activating and repressive histone marks, and RNA PolIII binding sites at the predicted *lincRNA-3q* gene promoter, in K562 cells (ENCODE data, UCSC genome browser). Transcript expression levels across various tissues and sequence conservation across species (bottom, as indicated). Primers used for *lincRNA-3q* RT-qPCR and sh target sequences are also indicated (top).

Figure S6: LincRNA-3q expression profiles in normal and cancer cells.

RT-qPCR-derived *lincRNA-3q* expression profiles in normal tissues (**A.**), leukemia and lymphoma cell lines (**B.**) and non-hematological cancer cell lines (**C.**), as indicated.

Figure S7: Knockdown of lincRNA-3q and BET inhibitor treatment in U937 cells.

A. Evaluation of U937 cell viability in non-transduced cells (NT), cells transduced with a non-targeting shRNA (shCtrl), or cells transduced with three different *lincRNA-3q* targeting shRNA (shA, B and C), respectively (left panel). Typical flow cytometry results, using Annexin V/propidium iodide for U937 cells transduced with non-targeting shRNA (shCtrl), or cells transduced with *lincRNA-3q* targeting shRNA B (right panel). **B.** RT-qPCR-derived *MYC* expression in U937 and CAL-1 cells treated with JQ1 at the time and doses indicated.

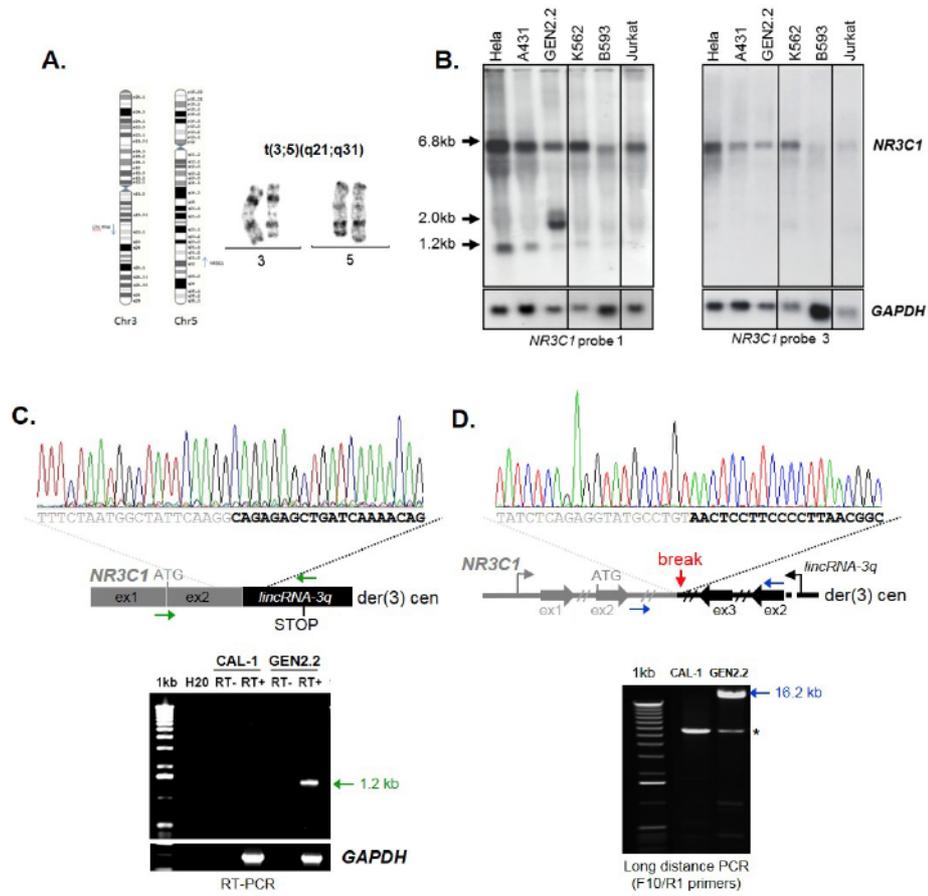


Figure S1

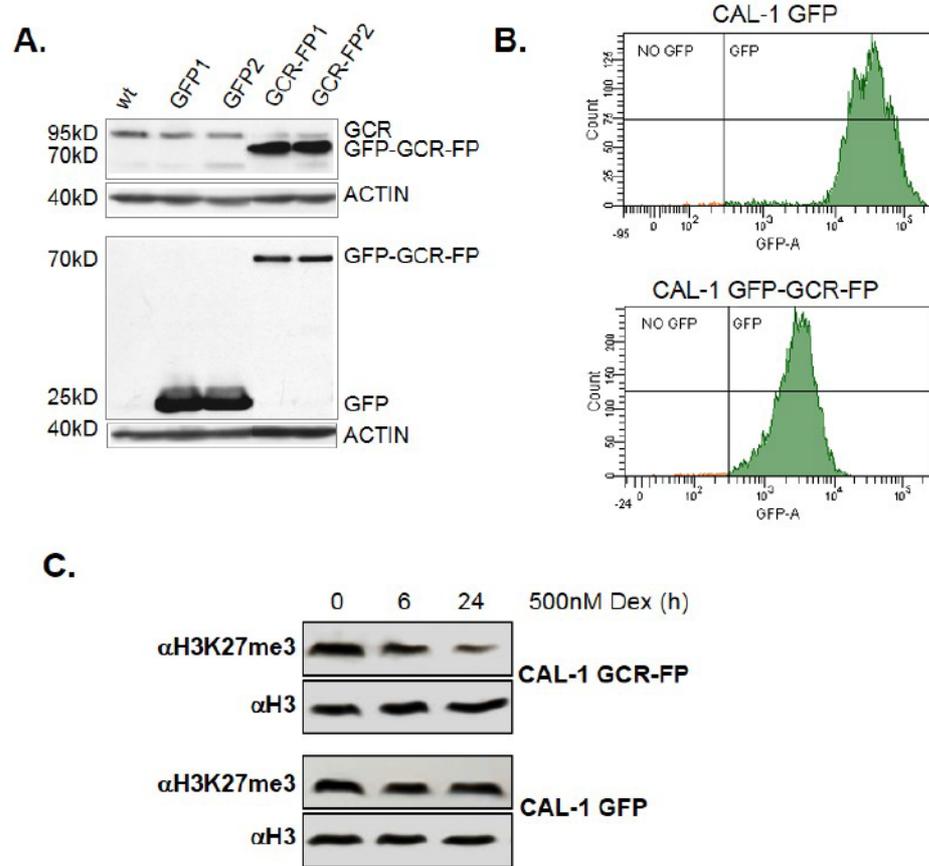
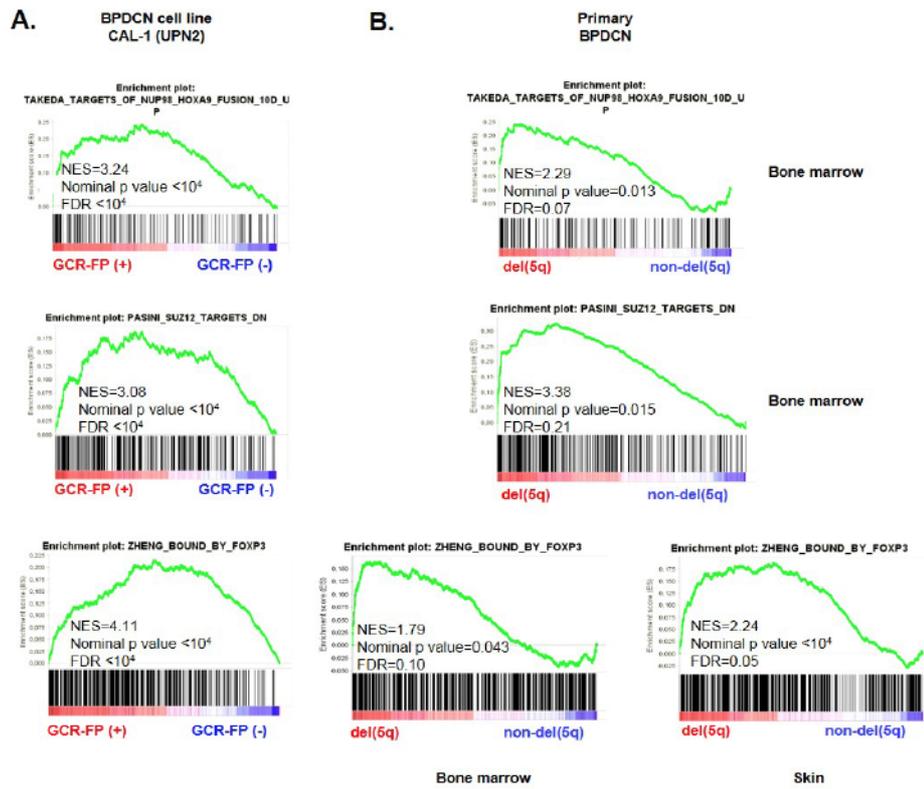


Figure S2



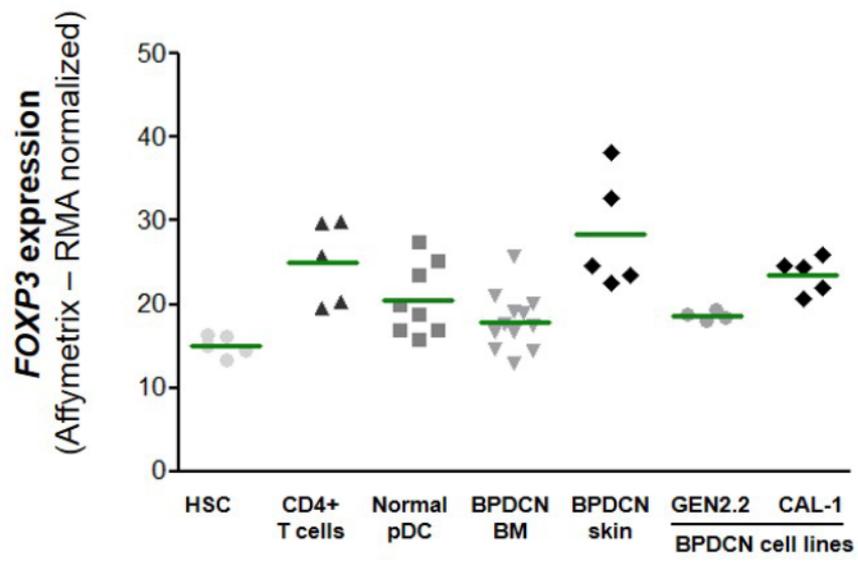


Figure S4

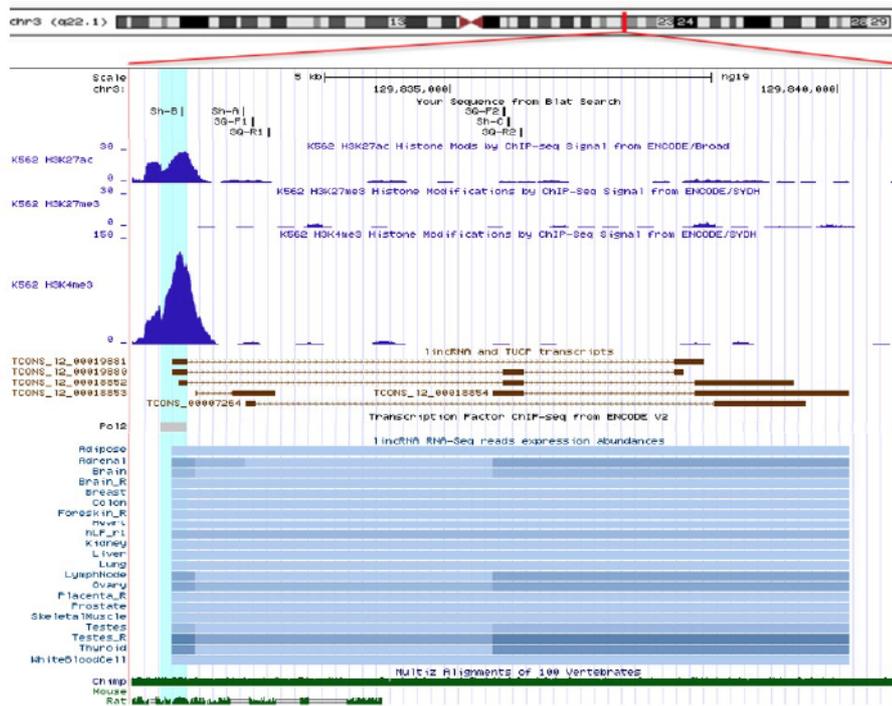


Figure S5

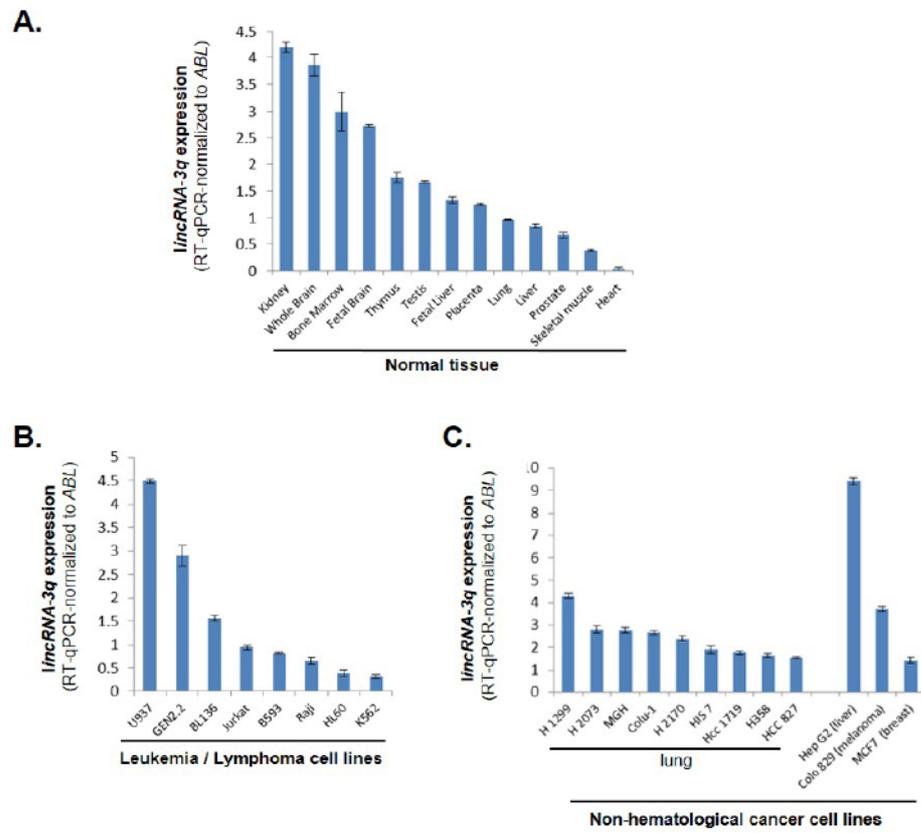


Figure S6

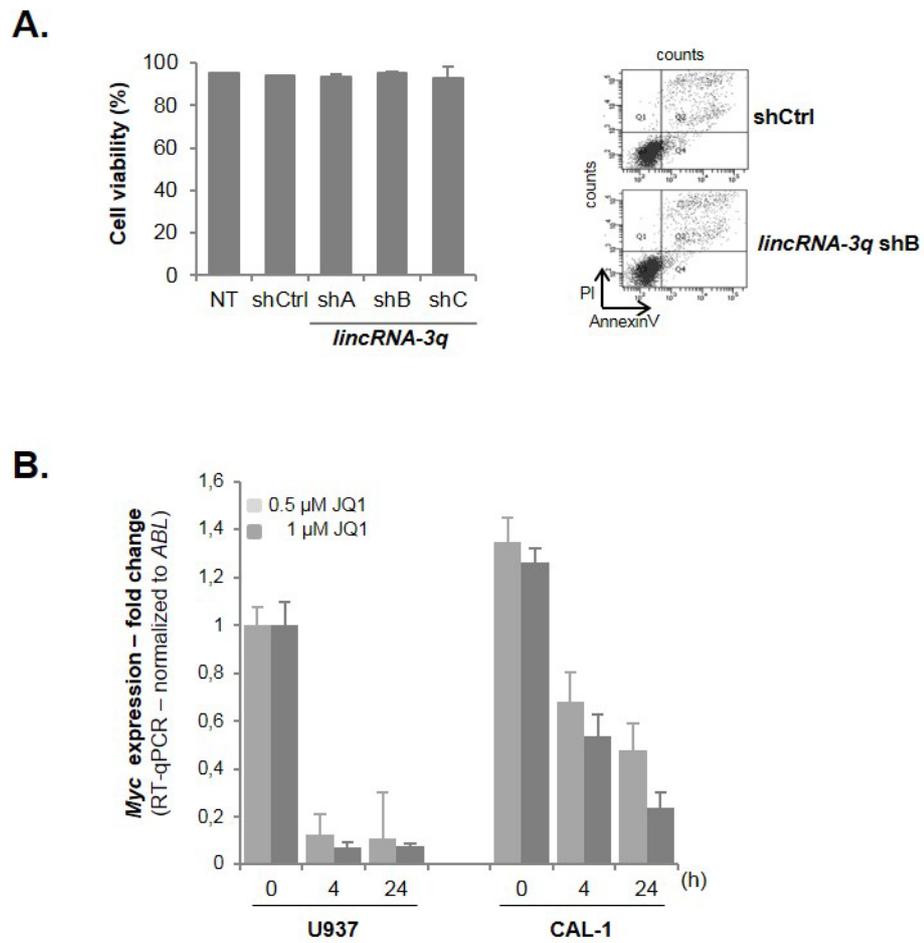


Figure S7

Table 51: Clinical characteristics, cytogenetic/FISH and molecular investigations in 47 cases of BPDCCN

UPN	Age(y) /sex	Clinical status	Survival (months)	Hematological disease	Extra- hematological sites	Prior History (time before diagnosis)	Source of cells for karyotype	% Blasts (BM) ¹	Karyotype	aCGRISH NR3C1 ^{2a}
	74/M	DOD		PB, BM	Skin	None	PB	100	49,XY,add(6)(q21),-8,2mar, r[8]#9,idel,t(15;16)(?q21;q21)[18]#49,idel,t(3; 5)(7q21.7;q31)15]ish wcp M-FISH 49,XY, 6,(6;8)(p21q24),r(12), 20#9,idel,inv(15)(q1?4q2?3),t(16;16) (q?:q?)#49,idel,t(3;5)(q?21;q?31)[13]	Split (subclone)
	76/M	DOD		PB, BM, LN, spleen	Skin	Skin, LN, spleen lesions (1m)	BM	100	45,X,- Y,add(1)(q43)add(5)(q3 74)t(6;8)(p21 :q24), del(6)(q23q25)add(11)(q22),+12, add(12)(p12)der(13;20)(q10;q10), add(16)(p13)add(18)(q21)add(19)(p13), der(21)(7;21)(q11;p12),der(22)[22qter- >22p12 8q11->8q24::6p21->6pter][20]	Normal
	72/M	DOD	26	BM	Skin	None	BM	86	49,XY,t(6;8)(p21q24),+11,+16,+18[19] 46,XY[1]	Normal
	41/M	DOD	18	BM, LN	None	None	BM	>50%	46,XY,t(6;8)(p21;q24)	Normal
	69/M	DOD		BM	Skin	None	PB	>50%	46,XY[15]	Normal
	78/M	DOD	29	BM	Skin	None	PB	>50%	46,XY[14]	Normal
	74/M	Alive	34	BM, LN	Skin	None	NA	>50%	ND	Normal
	64/F	DOD		PB, BM, LN	Skin	None	NA	>50%	ND	Deletion
	26/M	DOD	19	BM, LN	Skin	None	LN	70	43,XY,del(2)(q31),+4,del(6)(q15q23),-8,-9, t(13;22)(p12;q11),-22,+mar[2]146,XY[18]	Normal
10	51/M	Alive	60	PB, BM, LN, spleen	Skin	None	BM	48	46,XY,del(6)(q21q34),i(7)(q10), del(9)(q11q13)del(10)(q23q25)119#46,XY [1]	Normal
11	64/M	Alive	63	BM		RAEB1 (2y)	BM	47	46,XY[20]	Normal
12	62/F	DOD	29	PB, BM, LN, spleen	Skin	None	BM	91	47,XX,t(15)(p13 p14),del(5)(q13q15),+12, del(13)(q14)(14)146,XX[3]	Normal
13	58/M	Alive		BM	Skin	None	BM	44	46,XY[20]	Normal
14	67/M	DOD		PB, BM, LN	Skin, CNS	None	BM	89	46,XY,der(5)[5pter::Sp14->5q21::5q34 :12p11- >12p12],der(12)(5p15::5q31::12p12- >12qter)[13]1 44,sl,der(3)(3p)(p12q32),der(3)[8qter- >8q23::?3p24 >3qter],de1(4)(q21)der(6)(6;11)(q22;q11), t(7;9)(q11;p11),der(8)add(8)(p23) add(8)(q23),-9,-10,del(11)(q11),del(13)(q13), del(15)(q22)add(22)(q12)[p16]#6,XY[7]	Deletion
15	43/F	DOD	10	PB, BM	Skin	None	BM	40	46,XX[20]	Normal
16	75/M	Alive		BM	Skin	None	BM	91	43,Y,?der(X)(X;15)(p21;q14),1(3;12)(p21;q24)ad d(4)(q11),add(7)(p13),der(9)(1;9) (q12;p13),-10,-13,-15[17]#6,XY[5]	Normal
17	71/M	Alive	13	PB, BM	Skin	None	BM	97	47,XY,del(6)(q13q26),der(8)(8pter->8q24::8q21- >8q26::20p13- >20pter)del(12)(p12),der(20)(8;20)(q24 p13) [p16]#6,XY[4]	Normal
18	63/M	Alive		PB, BM	None	None	BM	95	47,XY,add(7)(p11)+20[8]#6,XY[1],ish i(7)(q10)(wcp7+),+20(wcp20+)	Normal
19	11/F	DO D	83	PB, BM, spleen	Skin	None	BM	90	46,XX,del(1)(q31),3,del(6q)add(12)(p13),- 13,+2mar<2>#46,XX<18>	Normal
20	82/M	DOD		None	Skin	None	BM	40	46,XY[20]	Normal
21	67/M	DOD DOD	11	PB, BM, LN	Skin	None	BM	82	43,XY,?del(5)(q21q22),add(12)(p11)- 13,?der(14)ins(14:?) (q24:?),-15,add(17)(p1 2),- 20[12]#3,sl- add(12)der(12;15)(q10;q10)[5]43,sd11, del(13)(q21q31)[2]#6,XY[1]	Normal
22	20/M	Alive		PB, BM	Skin	None	BM	45	45,XY,- 4,add(5)(p15)del(5)(q14q33)del(6)(q16q23) ,ad d(8)(q274),-9,der(13)ins(13;7)(q14;7),7-14- 15+21,+mars(3)/46,XY[17]	Deletion
23	74/M	DOD	10	PB, BM	Skin	RAEB1 (10y)	BM	20	46,XY[20],ish 8q24(MYCx2),11q23(MLLx2)	Normal
24	72/M	DOD		PB, BM	None	MPN (Sy)	BM	87	43,XY,t(2;6)(p21;q26)add(7)(q3?4), der(12 :18)(q10q10)del(13)(q13q21),- 15,der(16)(16;7)(q2?2q12),-17[15]	Deletion (subclone)

UPN	Age(y) /sex	Clinical status	Survival (months)	Hematological disease	Extra- hematological sites	Prior History (time before diagnosis)	Source of cells for karyotype	% Blasts (BM) ¹	Karyotype	aCGH/FISH NR3C1 ^{2,3}
25	82/M	Alive	18	PB, BM	Skin	None	BM	87	47,XY,+8[7],47,sl,t(7;14)(p14;q22)[17]/46,XY[1]. i,h,t(7;14)(wcp14+,IGH+wcp14+,IGH-)	Normal
26	65/M	DDD		BM	Skin	None	BM	74	41-46,XY,-3,-4,add(7)(p1?),-10,-12,-13,- 17,-2-Bmars,dmin[cp12]/45,XY,- 4,de12)t(4;12)(q17;q17?),dmin[6]/46,XY[7]	Deletion (subbne)
27	82/F	Alive		PB, BM	None	None	BM	88	45XX,add(1)(q23),-5,-6,-7,-9,-13+4mar[9]	Deletion
28	71/F	Alive	10	PB, BM, LN, spleen	Skin	None	BM	94	43-44,XX,der(8)t(8;12)(q22;q13),-9,-12,-t3[12],+1- 2mars[cp15]/46,XX[7]	Normal
29	12/F	Alive	42	BM, LN	Skin	None	BM	50	46,XX,t(38)(q28;q12)inv(8)(q12q24), t(5;12)(q23;p13)der(10)t(3;10)(q21;q25) t(3;8)inv(8)[4]/49,sl,t(5)(p10)- der(10),+10,der(14)t(3;14)(q21;p11)t(3;8) inv(8),+der(14)der(15)t(3;15)(q21;p11)(3;8) inv(8),+der(15)[50],sdl,t+20[2]/46,XX[8]	Normal
30	82/M	DOD		PB, BM, LN	Skin	None	BM	50	46,XY[20]	Normal
31	38/M	DOD	18	PB, BM	Skin	None	BM	60	44,X,-Y,del(9)(q11q31),- 13[2]/44,idem,add(4q?)add(5q?) [3]/46,XY[14]	Deletion
32	75/F	MD	MD	PB, BM	Skin	None	BM	90	45XX,de(5)(q13q34)inv(11)(p11q21), der(15)t(15;18)(?;?)-18	Deletion
33	82/M	MD	MD	PB, BM, LN	Skin	None	BM	84	46,XY[20]	Deletion
34	69/M	OOO		BM, LN	Skin	Colon cancer	BM	95	46,XY[20]	Normal
35	74/M	DOD	18	PB, BM	Skin	Skin lesions	BM	75	ND	Deletion
36	60/M	DOD	11	PB, BM, LN	Skin	None	BM	70	44,X,-Y,t(4;12)(?;?)-9,-13,+ma 9Y46,XY[18]	Normal
37	70/M	Alive	72	PB, BM	Skin	CMML	BM	85	44,X,-Y,del(6)(q23q25-27)x2,add(8)(q24), inv(9)(p11q13),t(12;15)(p10;p10),- 13[18]/46,XY,inv(9)(p11q13)x2[2]	Normal
38	59/M	DOD	15	PB, BM, LN	Skin	None	BM	95	46,XY[20]	Normal
39	61/M	DOD		PB, BM	Skin	None	BM	82	46,XY[20]	Normal
40	51/M	Alive	83	PB, BM, LN	Skin	None	BM	71	46,XY,del(8)(q11q22),del(9)(q11q13), del(10)(q12q13)[1]/46,XY,idem(7) (q10)[18]/46,XY[1]	Normal
41	15/M	Alive	54	PB, BM, LN	Skin	None	BM	62	43,XY,t(3;8),der(7)t(7;10)-9,der(12)t(5;12),13,- 19/86,idemx2	Normal
42	66/M	Alive	24	PB, BM, LN	Skin	None	BM	74	46,XY[20]	Normal
43	67/F	DOD		None	Skin, breast	None	NA	NA	NA	Normal
44	74/M	DOD	12	None	Skin	None	NA	NA	NA	Normal
45	60/M	DOD	22	BM	Skin	None	NA	NA	NA	Deletion
46	58/M	DOD	29	None	Skin	None	NA	NA	NA	Normal
47	7/F	Alive	20	None	Skin	None	NA	NA	NA	Deletion

DOD: dead of disease

PB: Peripheral blood, LN: lymph node, BM: bone marrow, CNS: central nervous system

RAEB: Refractory anemia with excess blasts, MPN: Myeloproliferative neoplasm, CMML: chronic myelomonocytic leukemia

NA = Not applicable, ND = Not done, MD = Missing data

bold: abnormal 5q

¹ estimated at >50% by aCGH for UPN4-8

² NR3C1 FISH performed in UPN 10-30

³ aCGH performed in UPN 1-9 and 31-47 (UPN 43-47 in skin biopsies, Djikman, 2008)

Table52: BACs used for FISH experiment

BAC	Chr.location	Gene	Genome coordinates	length (kb)
RP11-114A13	5q31.2	<i>MEF2L</i>	chr5:138,618,787-138,764,501	146
RP11-94G20	5q31.2	<i>MTOR</i>	chr5:138,508,691-138,688,954	180
RP11-114B22	5q31.2	<i>CYSTMIP/DNHBEGF</i>	chr5:139,536,924-139,713,162	176
RP11-261B11	5q31.2	<i>NRG2</i>	chr5:139,272,101-139,436,755	165
RP11-277D10	5q31.2	<i>NRG2</i>	chr5:139,666,321-139,860,054	194
RP11-301F04	5q31.2	<i>NRG2/PURAIGJP</i>	chr5:139,403,026-139,589,459	186
RP11-78P13	5q31.2	<i>PSD2/NRG2</i>	chr5:139,218,325-139,388,032	170
RP11-323J18	5q31.3	<i>SLC25A24P/SLC25A24</i>	chr5:140,664,083-140,827,682	164
RP11-429E15	5q31.3	<i>PODHB1 A/PODHB1</i>	chr5:140,400,738-140,549,204	148
CTD-202417	5q31.3	<i>COL18A3/SPPLN/SPPLN</i>	chr5:140,844,770-141,030,663	186
CTD-2040G12	5q31.3	<i>FCHS01/ARAP3</i>	chr5:141,030,664-141,111,527	81
CTB-88J14	5q31.3		chr5:141,740,973-141,883,180	142
CTD-2185H17	5q31.3	<i>ARHGAP28/NR3C1</i>	chr5:142,525,913-142,761,634	236
RP11-278J6*	5q31.3	<i>NR3C1</i>	chr5:142,680,739-142,816,857	136
CTB-163B16	5q32	<i>PREL/02</i>	chr5:144,861,294-144,945,294	84
CTD-2277F12	5q32	<i>SPINK5/SPINK14</i>	chr5:147,515,884-147,570,458	55
RP11-331K21	5q32	<i>ABLIM3</i>	chr5:148,461,756-148,580,284	119
RP11-34P1	5q33.3	<i>FABP6/CCNJL</i>	chr5:159,651,182-159,796,906	146
RP11-117L6	5q35.1	<i>NPM1/FGF18</i>	chr5:170,829,745-170,882,555	53

• :split signal

Table 53: Long distance PCR primers for breakpoint mapping and cloning

Primer name	Forward primer	Genome coordinates
5q primers		
LD-NR3C1-F1	GGCAATGATGATGCGGCTCT	chr5:142,777,288-142,777,269
LD-NR3C1-F2	CACCCGAGAGGTGGGGCTCT	chr5:142,772,700-142,772,681
LD-NR3C1-F3	TCCCCAGCAGCATGGAGAGC	chr5:142,768,779-142,768,760
LD-NR3C1-F4	TGCCTCCCCCTCCCTCAGTT	chr5:142,768,739-142,768,720
LD-NR3C1-F5	GCAGAGCCACCAGGGCAGAG	chr5:142,764,291-142,764,272
LD-NR3C1-F6	CCTGGCCCCCAATTTTCATCC	chr5:142,757,539-142,757,520
LD-NR3C1-F7	TCCAGTCTCCACCGCACCT	chr5:142,756,462-142,756,443
LD-NR3C1-F8	TGCTCGCTGGATGCCCTTTC	chr5:142,753,382-142,753,363
LD-NR3C1-F9	TCAGCTGTGGCCCCCTTGACC	chr5:142,748,435-142,748,416
LD-NR3C1-F10*	TTGCCCCCTTCCGCTCCTTC	chr5:142,743,537-142,743,518
LD-NR3C1-F11	TTGGGGTGGTGAACGGGAAG	chr5:142,737,741-142,737,722
LD-NR3C1-F12*	CCAGTTGGCTTCAGGCAGCA	chr5:142,735,491-142,735,472
LD-NR3C1-F13	CATGCCACAACACAAAAGGGACA	chr5:142,731,163-142,731,141
LD-NR3C1-F14	CCCAAGGCCTGGGTGTGGAT	chr5:142,729,104-142,729,085
LD-NR3C1-F15	GCCGAAACCCAGGGAAGCTG	chr5:142,722,865-142,722,846
LD-NR3C1-F16	AGCCATGGGGCCATTTACCG	chr5:142,720,920-142,720,901
LD-NR3C1-F17	TGGAAGGGCCTCGGCATGTA	chr5:142,714,766-142,714,747
LD-NR3C1-F18	TGGAAGGGCGCAAGAACCA	chr5:142,712,692-142,712,673
LD-NR3C1-F19	CCCCTGCCAGGCAACAGTCA	chr5:142,705,789-142,705,770
LD-NR3C1-F20	TCATGATTGGAGGCAGGCACA	chr5:142,702,041-142,702,021
LD-NR3C1-F21	GCCCGTATGACCGCATCCAG	chr5:142,696,110-142,696,091
LD-NR3C1-F22	CCGCATCCAGGGGTGATGAT	chr5:142,696,100-142,696,081
Primer name	Reverse primer	Genome coordinates
3q primer		
3q-R1	CCAGGGATGGGTCTGGGTGA	chr3:129,832,386-129,832,405

*: breakpoint amplification

used for breakpoint cloning

Table 54: RACE-PCR and RT-qPCR primers

RACE-PCR		
Primer name	Primer sequence	Localisation
NR3C1-3'RACE	TCTGGTTTTGTCAAGCCCCAGT	NR3C1 exon 2
NR3C1-5'RACE	TCCTGCTGTTGAGAAAGGGATGCTG	NR3C1 exon 2

RT-PCR (fusion transcript isolation)	
Primer name	Primer sequence
NR3C1-Forw	ATGGACTCCAAAGAATC
3Q-R1-Rev	TCAACGGTTTCTGGGTCTG

RT-qPCR (quantitative PCR)	
Primer name	Primer sequence
3Q-F1	GGGCACTGCTTTGCTTTTGCTGT
3Q-R1	CTGAACCAACCTAGATTAATATTTTAGTATA
3Q-F2	AAATTGCCTGTTTGCTCCAC
3Q-R2	CAGAGTTTCTGAGGCCTTGC

Table 55: PCR primers for fusion transcript molecular cloning and sequencing

pcDNA3.1(+) Fusion Transcript cloning primers		
Primer name	Primer sequence	Restriction site
ATG-NR3C1-F1	(TAGCGGA TCCACC)A TGGACTCCAAGAA TC	BamH1
3Q-R1	(TAGCGAATTCA)TCAACGGTTTCTGGGTCTG	EcoR1
pEGFP-C1 Fusion Transcript cloning primers		
Primer name	sequence	Restriction site
Ntag-NR3C1-F1	(TAGCGGATCC)GACTCCAAGAA TC	BamH1
3Q-R1	(TAGCGAATTCA)TCAACGGTTTCTGGGTCTG	EcoR1
PCR pSFFV-HIV-(mRFP)-wPRE GFP-Fusion Transcript cloning primers		
Primer name	sequence	Restriction site
BamHI-ATG-EGFP-FT-F1	(TAGCGGA TCCACC)A TGGTGAGCAAGGGC	BamH1
Xho1-TGA-EGFP-FT-R1	(CTCGAGAATTCA)TCAACGGTTTCTGGGTCTGAG	Xho1
Sequencing primers for pSFFV-HIV-GFP-Fusion Transcript vector		
Primer name	sequence	
GFP-TAAseq-F1	AAGATCCGCCACAACATCG	
GFP-ATGseq-R1	CTTCTCGTTGGGGTCTTTGC	
HIV-SFFV-seq-R1	AATGAAAGCCATACGGGAAG	

Table 56: PCR primers for Northern Blot probes amplification

Primer name	Forward primer	Reverse primer
NR3C1_S_1/NR3C1_AS_1*	GCCAGAGTTGATATTCAGT	GGTACTCACACCATGAACAG
NR3C1_S_2 / NR3C1_AS_2	CTGTTTCATGGTGTGAGTACC	CTCAGAGGAAACATACAGC
NR3C1_S_3 / NR3C1_AS_3	GCTGTATGTTTCCTCTGAG	CCATTCTTATTAAGGCAGTC
NR3C1_S_4 / NR3C1_AS_4	GACTGCCTTAATAAGAATGG	GCCATATAGCCATTGCAAAAATAG

* :anormal transcript detected

Table 57: Sequence of short-hairpin 3q cloned in pLK0.1 vector (Addgene)Designed using DSIR algorithm (<http://biodev.cea.fr/DSIRwebsite>)

Primer name	Hairpin sequence (gel -> EcoRI cloning)
Sh-A sens	CCGGTCGGGCTGAGATGTTGCATCAACTGCAGATGTATGCAACATCTCAGACCCTTTTG
Sh-A AS	AATTCAAAAGGGT CTGAGATGTTGCATCAACTGCAGAT GTATGCAACATCTCAGACCCGA
Sh-B sens	CCGGTCGCCTTTGGAGTGTACCAAACTGCAGAGTTGGTGACTCCAAAGGCT TTTTG
Sh-B AS	AATTCAAAAGCCTTTGGAGT GTCACCAAACTGCAGAGTTGGT GACTCCAAAGGCG A
Sh-C sens	CCGGTCGGAGCAGTCCATCAAGAATGAACTGCAGATCATTCTTGAT GGACTGCTCCTTTTG Sh-
CAS	AATTCAAAAGGAGCAGTCCA TCAAGAATGAACTGCAGATCATTCTTGATGGACTGCTCCGA

Source : mRNA chosweyby.cApr07

Loop (st1) : CTGCAG

Table 58: PCR primers for qChIP

Gene promoter	Forward primer	Reverse primer
<i>HOXA5</i>	GAGAGACTGGGCTCTGTTGG	AGGGGGTCTAAGAGCTGGAG
<i>HOXA9</i>	GCGAATGTCCCCTAATCAGA	AGACAAGGCCTCGGTTATGA
<i>HOXA11B</i>	GAGTTGAAGCCGTGGATGT	TCGGAAGTGACCATGAATGA

General discussion and perspectives

In a clinically guided approach focused on functional genomic characterization of a novel t(3;5)(q21;q32) translocation we have uncovered novel disease mechanisms centering on the glucocorticoid receptor and a novel BET-inhibitor sensitive, long, non-coding RNA in the pathogenesis of BPDCN.

***NR3C1* deletion is associated with poor prognosis and drives abnormal EZH2 signaling in BPDCN**

Our initial interest in our BPDCN case presenting a t(3;5) translocation stemmed from its high potential for discovery, by functional genomic analysis, of 5q-linked disease mechanisms in BPDCN. Chromosome 5q deletions, outside of the 5q- syndrome, have been demonstrated to be associated with increased risk of leukemic transformation in MDS and with very poor prognosis in AML (Byrd et al., 2002, Grimwade et al., 2001). Chromosome 5q loss is also prevalent in MDS and AML arising after previous cancer treatment with alkylating agents or radiotherapy (Bhadri et al., 2012, Pedersen-Bjergaard et al., 1990) suggesting strongly an important role for affected genes in 5q locus in leukemogenesis and leukemia progression. This hypothesis is supported by numerous studies including the recent identification of a TRAF6-mediated NF- κ B signaling survival network driven by 5q deletion in AML / MDS (Fang et al., 2014).

Chromosome 5q deletion is one of the most recurrent chromosomal abnormalities observed in BPDCN (Jardin et al., 2009, Leroux et al., 2002, Petrella et al., 2005). The genes and gene-networks involved in del(5q) in BPDCN have not been investigated until this work. Our molecular cytogenetic and gene cloning efforts of the t(3;5) pointed to *NR3C1*, as an important target gene in 5(q)-linked BPDCN pathogenesis.

Indeed, the *NR3C1* gene underwent recurrent homozygous deletion in 13 of 17 (76%) 5q-rearranged BPDCN cases. This included 1 case with focal *NR3C1* deletion in addition to the index case of reciprocal chromosomal translocation. The latter resulted in expression of a truncated GCR isoform, lacking the DNA and ligand binding domains. Importantly, our clinical data demonstrated that the loss of this gene is associated with a very poor outcome in BPDCN patients. In this work we demonstrated that glucocorticoid receptor expression levels are decreased in primary BPDCN samples presenting del(5q), compared to controls, suggesting a haploinsufficiency mechanism for GCR in del(5q) cases.

Luciferase reporter assays of GCR activity, in the presence of the GCR fusion protein (GCRFP) resulted in reduced activity of GCR transcriptional transactivation, suggestive of functional interference and a mechanism mimicking *NR3C1* haploinsufficiency in these cells. *In vitro* and *in vivo*, *NR3C1* knock-down experiments to GCR levels similar to those found in del(5q) cases (i.e. 50%) are in process to further confirm these observations.

Perturbed GCR signaling induces treatment resistance in BPDCN

Cortico-resistance is a common feature in leukemic disorders and it has been demonstrated that this can manifest as early cortico-resistance or cortico-resistance following prolonged treatments ultimately leading to relapse. *NR3C1* deletion has been associated to acquired corticoresistance in B-ALL (Mullighan et al., 2008) We assessed the biological consequence of truncated GCR to treatment response and discovered that it confers resistance to combination therapy with dexamethasone and etoposide. Consistent with this, gene set enrichment analysis (GESA) on dexamethasone treated CAL-1 BPDCN GCR-FP-overexpressing cells compared to controls demonstrated a perturbed GCR signaling, accompanied with cortico-resistance signatures in B-ALL (Holleman et al., 2004).

Perturbed *EZH2* and *FOXP3* regulatory networks, a hallmark of 5q alterations targeting *NR3C1* gene in BPDCN

A striking finding in our gene expression profiling analysis in dexamethasone treated CAL-1 GCR-FP-overexpressing cells compared to controls was the discovery that functional deficiencies in the GCR signaling pathway provoked an *EZH2* loss of function phenotype (Kondo et al., 2008, Takeda et al., 2006). This was confirmed experimentally by direct demonstration of global loss of H3K27 trimethylation in the affected cells. Furthermore, this was associated to loss of H3K27me3 at promoters of genes within the *HOXA* locus which is critical to hematopoiesis (Khan et al., 2013) and frequently deregulated in myeloid malignancies consequent to mutations affecting components of the PRC2 complex (Abdel-Wahab et al., 2012, Lawrence et al., 1996). Importantly the *EZH2* loss of function signature was also evident in primary BPDCN patient transcriptomic data when classed according to *NR3C1* deletion status.

Further functional investigations, such as co-immunoprecipitation experiments would be needed to elucidate the precise molecular mechanisms responsible.

A second striking finding was identification of altered *FOXP3* network signatures in our experimental system for *NR3C1* deletion and in primary BPDCN patients with *NR3C1* deletion versus non-deletion. Interestingly *FOXP3* repressor functions appeared to be specifically compromised. It has been demonstrated that transcription factor Foxp3 (forkhead box P3), is restricted in its expression to a specialized regulatory CD4⁺ T- cell subset (T_R) which maintain immune tolerance, suppress fatal inflammation, and controls TR lineage development and commitment through suppression of alternative lineage choices (Arvey et al., 2014, Fontenot and Rudensky, 2005, Zheng et al., 2007). Recently *FOXP3* expression was demonstrated not to be restricted to the lymphocyte lineage and it has demonstrated to be expressed by some non-lymphoid normal and cancerous cells (Martin et al., 2010). Interestingly we observed expression of *FOXP3* in normal pDC and BPDCN cell lines (Gen2.2 and CAL-1) and primary BPDCN samples, albeit at low levels. This suggests a regulatory function for *FOXP3* in pDCs and that it is deregulated in leukemic samples.

FOXP3-bound sites in CD4 T helper cells have been demonstrated to present diminished chromatin accessibility and selective deposition of histone H3 trimethylated at Lys27 (H3K27me3), and this is attributed to the capacity of FOXP3 to directly interact and recruit EZH2 to cognate sites (Arvey et al., 2014). Thus loss of the repressive function of FOXP3 might occur as a consequence of a feed forward inhibitory loop driven by *NR3C1* / GCR haploinsufficiency in BPDCN.

***lincRNA-3q* a novel leukemia driver in BPDCN and AML ?**

A second major finding from our clinically guided approach to BPDCN pathogenesis was the discovery of a novel, nuclear *lincRNA* gene with potent anti-proliferative activities in BPDCN and AML.

This *lincRNA* named here, *lincRNA-3q*, was ectopically expressed in of our BPDCN primary cases and in high-risk AML, particularly those with 3q abnormalities (inversions or translocations). This is of keen interest because AML with *inv(3)/t(3;3)* is known to be associated with aberrant expression of the stem-cell regulator gene *Ecotropic viral integration site 1 (EVII)* located in 3q26. This gene encodes a member of the SET/PR domain transcription factors. Chromosomal rearrangements affecting chromosome 3q26 activate *EVII* expression especially in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Goyama et al., 2008). It has been demonstrated that this occurs consequent to repositioning of a distal *GATA2* enhancer to the *EV1* gene resulting in its ectopic activation (Groschel et al., 2014). This observation is of interest, since this “super enhancer” is located approximately 1Mb centromeric of *lincRNA-3q* and therefore in cases with 3q rearrangements, the *GATA2* enhancer could also impact the *lincRNA-3q* expression. Further analysis would be necessary to verify this hypothesis by treatment with BET inhibitors which can decommission the *GATA2* super enhancer also show marked suppression of *lincRNA-3q*. Short hairpin RNA (shRNA) of *lincRNA-3q* caused a significant G1/S cell cycle arrest in U937 AML cells (U937). GEP analysis on KD and control cells followed by GSEA revealed this to be linked to suppression of multiple proliferation genes many of which are driven by E2F-dependent promoters and to upregulation of E2F7/8, an inhibitory E2F family member.

The E2F transcription factors function in cell cycle control and are intimately regulated by *RB* (Chen et al., 2009). In mammals, the *E2F* family comprises eight genes (*E2F1–8*), which give rise to nine distinct proteins. Deregulated E2F activity is observed in the vast majority of human tumors and occurs through several different mechanisms. These proteins are categorized into subfamilies on the basis of their transcriptional activity, structure and interaction with Rb family members. The “activator E2Fs” including E2F1, E2F2 and E2F3A, implicated mainly as gene expression activators and interact only with RB. E2F4–8 largely function in the repression of gene expression and are generally referred to as the “repressor E2Fs”. In the subfamily of repressor E2Fs, E2F4 and E2F5 repress gene expression in an RB family-dependent manner, whereas E2F6–8 exert transcriptional repression through distinct, Rb-independent, mechanisms. Generally speaking, activity of the activator E2Fs is required for cell proliferation, whereas the repressor E2Fs function in cell cycle exit and differentiation (Figure 14) (Polager and Ginsberg, 2009).

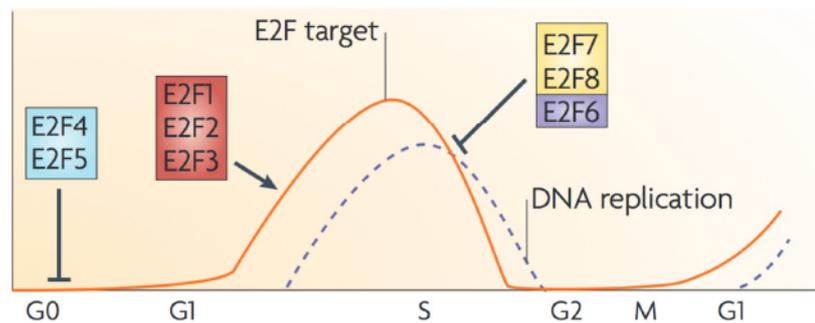


FIGURE 14: In quiescent (G0) cells, the ubiquitously expressed E2F4 and E2F5 associate with pocket proteins and other corepressors to maintain repression of E2F-responsive genes (line labeled E2F target) that promote entry into the G1 phase of the cell cycle. Upon mitogenic stimulation, the sequential phosphorylation of RB by activated cyclin-dependent kinases results in the loss of RB function, release of E2F repressors and the accumulation of newly synthesized free E2F1, E2F2 and E2F3 late in G1 phase. Together, these events initiate a transcriptional program driving cells into S phase (dashed line labeled DNA replication). This G1/S-specific transcriptome is then attenuated upon completion of S phase in G2 by the action of the repressors E2F6, E2F7 and E2F8, which are thought to function independently of RB and RB-related proteins. *From (Chen et al. 2009, Nat Rev Cancer)*

This suggests that *lincRNA-3q* might control the transcriptional activity of E2F-bound genes. In order to confirm this, E2F-luciferase reporter assays in the presence or absence of a recombinant *E2F* and small interfering RNA (siRNA) targeting *lincRNA-3q* are underway. Likewise, *in vivo* leukemia growth assays are planned to determine the impact of this lincRNA in leukemia progression *in vivo*.

A major challenge in the future will be molecular dissection of how *lincRNA-3q* might influence E2F activity. The first possibility is that lincRNA-3q directly binds E2F family members and either regulates their activity or stability. Indeed, previous studies have suggested that nuclear lncRNAs are associated with chromatin modifying complexes such as PRC2 (Khalil et al., 2009) and MLL (Wang et al., 2011). For example, HOTAIR, which is known for its role in promoting tumor metastasis (Gupta et al., 2010), binds to EZH2/PRC2 and LSD1 to coordinate their function in epigenetically regulating gene expression (Tsai et al., 2010) and HOTTIP, regulates *HOXA* expression by interacting with the activating H3K4 methyl transferase, MLL1 complex on *HOXA* gene promoters (Wang et al., 2011). Hu et al by performing a genome-wide survey on somatic copy-number alterations (SCNAs) of long noncoding RNA (lncRNA), identified an oncogene, *focally amplified lncRNA on chromosome 1 (FAL1)*, whose copy number and expression are correlated with outcomes in ovarian cancer. FAL1 binds to epigenetic repressor BMI1 and regulates its stability, controlling the expression of a number of genes including *CDKN1A* (Hu et al., 2014).

Since our *lincRNA-3q* KD resulted in the repression of E2F target genes, we propose the following model for *lincRNA-3q* in leukemogenesis. Under physiological conditions *lincRNA-3q* is expressed at low levels, which would favor normal G1/S progression through balanced activity of activatory and inhibitory E2F members. In contrast, in leukemia, overproduction of *lincRNA-3q* might drive favor uncontrolled G1/S progression by constraining activity of inhibitory E2F members such as E2F7/8 (Figure 15).

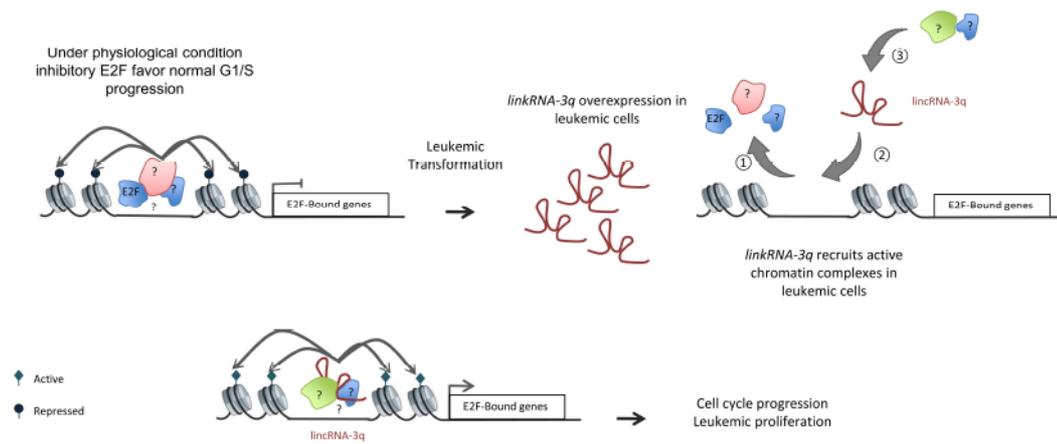


FIGURE 15: A proposed model of the functional consequence of *lincRNA-3q* expression in leukemic cells

Overview of experimental strategies for assessing nuclear lncRNA function

The above direct interaction model could be assessed by performing RNA pull down assays, using *in vitro* transcribed biotinylated *lincRNA-3q* or RNA immunoprecipitation assay (RIP) using an anti-E2F antibody for example. RIP is an antibody-based technique used to map RNA–protein interactions *in vivo* by immunoprecipitating the RNA binding protein (RBP) of interest together with its associated RNA and quantifying interaction with the transcript of interest versus control.

The genome is regulated by trans-acting factors that bind to specific loci in chromatin and in addition to proteins, as mentioned in the introduction, long non-coding RNAs can act on chromatin at sites distant from where they are transcribed (Simon, 2013). Numerous techniques that allow the identification “sans a priori” of the protein partners and chromatin-bound sequences of uncharacterized lncRNAs, such as our *lincRNA-3q* have emerged in recent years. These include RAP (RNA antisense purification) (Figure 16), CHART (capture hybridization analysis of RNA targets) (Figure 17) and ChIRP (chromatin isolation by RNA purification) (Figure 18), techniques.

These techniques are all based on the same principle, which is to use biotinylated oligonucleotides complementary to the RNA of interest, and pull down associated proteins, or more accurately, chromatin-bound RNA. This is then followed by or mass

spectrometry and/or NGS to identify respectively the proteins associated with the RNA and the genomic locations at which those interactions occurred (Engreitz et al., 2013).

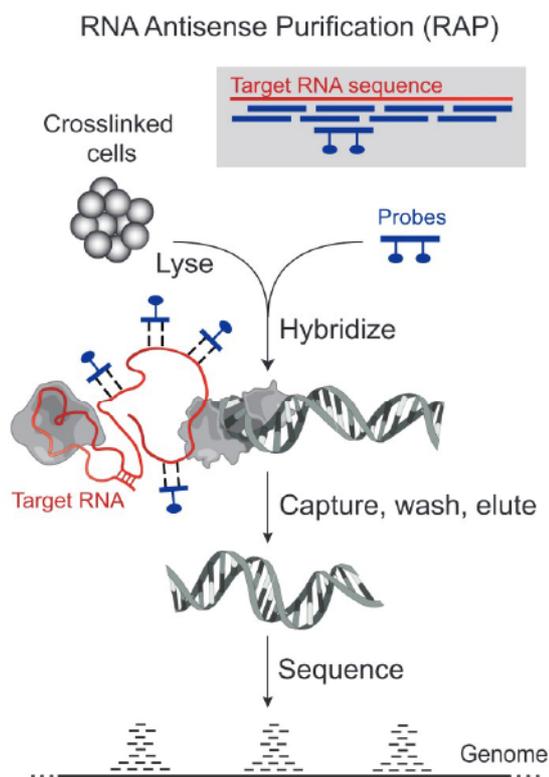


FIGURE 16: Schematic diagram of RAP. Biotinylated probes (blue) are hybridized to the target RNA (red) crosslinked to proteins and DNA. Gray inset: 120-mer antisense capture probes are designed to tile across the entire target RNA. From (Engreitz et al. 2013, *Science*)

An alternative technique encompassing many features of the techniques mentioned above is domain-specific chromatin isolation by RNA purification (dChIRP), which is a scalable technique that will dissect pairwise RNA-RNA, RNA-protein and RNA-chromatin interactions at the level of individual RNA domains in living cells (Figure 18) (Quinn et al., 2014).

dChIRP can reveal lncRNA architecture and function with high precision and sensitivity (Quinn et al., 2014). Therefore in order to characterize the chromatin-binding pattern and interacting protein factors of *lincRNA-3q*, dChIRP would be a highly informative technique.

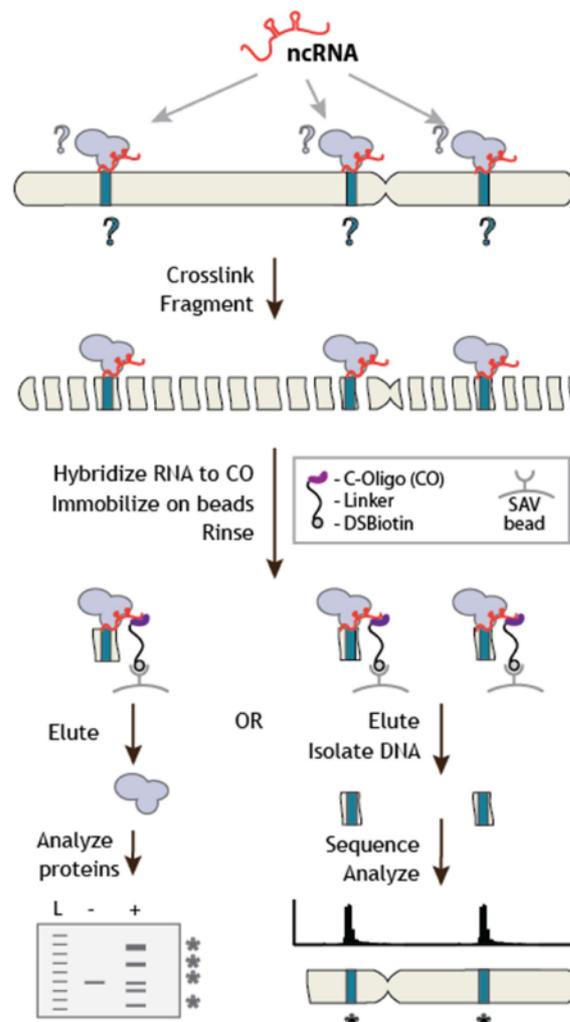


FIGURE 17: CHART is a hybridization-based strategy that uses complementary oligonucleotides to purify the RNA together with its targets from reversibly cross-linked extracts. The cartoon here shows the scenario where the RNA is bound in direct contact with the DNA together with proteins, but other configurations are also possible. CHART-enriched material can be analyzed in various ways; the two examples depicted here are (Left) sequencing the DNA to determine genomic loci where the RNA is bound and (Right) analyzing the protein content by Western blot analysis. From (Simon et al, *Proc Natl Acad Sci*, 2011)

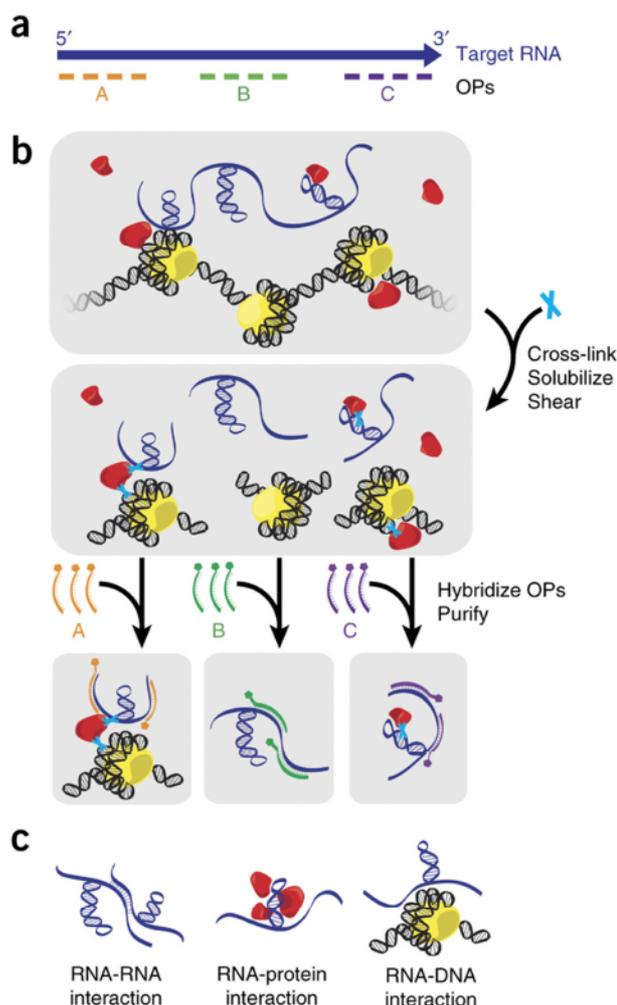


FIGURE 18: dChIRP uses antisense oligonucleotides to purify specific RNA domains and associated RNAs, proteins and chromatin. (a) dChIRP oligonucleotide design strategy. Biotinylated antisense oligonucleotide pools (OPs) are designed to tile specific regions of the target RNA. (b) dChIRP workflow. To prepare chromatin, whole cells are cross-linked to preserve protein–nucleic acid interactions. Sonication is used to solubilize the nuclear fraction and shear nucleic acids. Next, the chromatin is subdivided into equal samples. OPs are added to each sample, which hybridize to the targeted RNA fragments. The biotinylated oligonucleotides, RNA targets and cross-linked biomolecules are then purified on magnetic streptavidin beads, and unbound material is washed away. (c) RNA-, protein- and DNA-sensitive modalities of dChIRP. RNA, protein and DNA fractions are extracted from each dChIRP sample. Intra- or intermolecular RNA-RNA, RNA-protein and RNA-DNA interactions may be measured by RT-qPCR, immunoblotting, and qPCR or sequencing, respectively. *From (Quinn et al. 2014, Nat Biotechnol)*

***lincRNA-3q* BET-inhibitor sensitivity**

Molecular profiling approaches have revealed multiple abnormalities in the genes affecting DNA methylation and chromatin remodeling complexes in cancer cells. Today epigenetic regulators represent a promising new class of therapeutic targets for cancer (Xu et al., 2012). In order to propose a drug capable of targeting *lincRNA-3q*, we assessed the inhibitory effect of JQ1, a BET inhibitor molecule on *lincRNA-3q* expression levels. JQ1 treatment resulted in suppression of *lincRNA-3q* in a dose and time dependent manner in BPDCN (CAL-1) and AML (U937) cell lines. It is noteworthy that JQ1-mediated BET inhibition might partly be due to *MYC* suppression through disruption of the ability of BET proteins to bind *MYC* locus (Zuber et al., 2011). Since the suppressive effect on *lincRNA-3q* appeared as soon as 4 hours after beginning the treatment, we assumed a direct inhibitory effect of JQ1 on the expression of this gene. Although characterization of the precise mechanism involved requires further investigation (BET protein binding to the *lincRNA-3q* locus itself?), these results suggest a new-targeted therapeutic strategy that could be applied to BPDCN.

In conclusion the molecular characterization and cytogenetic analysis of our BPDCN cohort have permitted the identification of two important genes in leukemogenesis of BPDCN. Our functional and genomics analysis highlighted the deviant GCR signaling, and unscheduled activation of *lincRNA-3q*, driving in part the deregulation of chromatin and transcription factor networks controlling treatment resistance and malignant reprogramming in the dendritic cell lineage. Further studies will be needed to fully leverage these findings for treatment innovation in BPDCN and other myeloid and lymphoid cancers that share biological features with this disease.

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