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# The proto-oncogene c-Kit inhibits tumor growth by behaving as a dependence receptor

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# The proto-oncogene c-Kit inhibits tumor growth by behaving as a dependence receptor

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## **ABSTRACT**

c-Kit has been generally considered as a receptor tyrosine kinase and a proto-oncogene, whose upregulation and mutation lead to tumor progression through its kinase activity. Clinically, drugs targeting the kinase activity of c-Kit, such as Imatinib (Gleevec), have been widely used to treat patients with c-Kit related diseases. While the role of c-Kit as a proto-oncogene is of no doubt, some research reports and database analysis do not fit well the tumor promoting role of c-Kit, indicating a possible different role of c-Kit in cancer. Here, we show that c-Kit belongs to the dependence receptor family, similarly to other receptor tyrosine kinases such as MET, RET and TrkC. In the absence of its ligand SCF (stem cell factor), instead of staying inactive, c-Kit triggers apoptosis, which can be enhanced by silencing its kinase activity. Besides, we have shown that c-Kit is able to bind and activate caspase-9. Moreover, similarly to other dependence receptors, c-Kit is also cleaved by caspases-like protease at aspartic acid residue D816, which is crucial for its pro-apoptotic activity. The mutation of D816 site inhibits the c-Kit/caspase-9 binding and silences the pro-apoptotic activity of c-Kit. Of interest, c-Kit D816 mutation is one of the most common mutations of this receptor in many c-Kit related cancers and it promotes resistance against Gleevec treatment. We also show that overexpression of kinase mutated c-Kit is able to inhibit tumor growth in animal models, while the mutation of D816 site impairs the tumor suppressing activity. Furthermore, we develop a tool to block the SCF/c-Kit interaction, which unleashes the pro-apoptotic activity of c-Kit in cancers expressing this receptor. By using the pro-apoptotic activity of c-Kit, in combination with kinase inhibitors like Gleevec, we propose a novel therapeutic strategy. In conclusion, we demonstrate that c-Kit is a member of dependence receptor family, harboring intrinsic pro-apoptotic activity, which can be used as an alternative tool in cancer treatment.

## RESUME

c-Kit est généralement considéré comme un récepteur à tyrosine kinase et comme un proto-oncogène, dont la surexpression et la mutation conduisent à une progression tumorale médiée par son activité kinase. En clinique, les traitements ciblant l'activité kinase de c-Kit, comme l'Imatinib (Gleevec), ont été largement utilisés pour traiter les patients atteints de maladies liées à c-Kit. Alors que le rôle de c-Kit comme proto-oncogène ne fait aucun doute, certaines études et analyses de bases de données diffèrent avec l'idée d'un rôle pro-tumoral de c-Kit, laissant penser à un rôle différent de c-Kit dans le cancer. Ici, nous montrons que c-Kit appartient à la famille des récepteurs à dépendance, de la même façon que d'autres récepteurs de la famille des tyrosine kinases tel que MET, RET et TrkC. En absence de son ligand Stem Cell Factor (SCF), au lieu de rester inactif, c-Kit déclenche l'apoptose, qui peut être renforcée par l'inactivation de son activité kinase. En parallèle, nous montrons que c-Kit est capable de se lier à la caspase-9 et de l'activer. De plus, à la manière d'autres récepteurs à dépendance, c-Kit est aussi clivé par des protéases de type caspase sur son résidu acide aspartique D816, qui est nécessaire à son activité pro-apoptotique. La mutation du site D816 inhibe l'interaction entre c-kit et la caspase-9 et invalide l'activité pro-apoptotique de c-Kit. De façon intéressante, la mutation D816 est l'une des mutations les plus communes de ce récepteur dans la plupart des cancers liés à c-Kit, et cette mutation favorise la résistance au traitement Gleevec. Nous montrons aussi que la surexpression de c-Kit invalidé pour son activité kinase est capable d'inhiber la croissance tumorale dans des modèles animaux, alors que la mutation du site D816 empêche son effet suppresseur de tumeur. En outre, nous avons développé un outil permettant de bloquer l'interaction entre SCF et c-Kit, déclenchant l'activité pro-apoptotique de c-Kit dans les cancers positifs pour ce récepteur. En utilisant l'activité pro-apoptotique de c-Kit, en combinaison avec des inhibiteurs de kinases comme le Gleevec, nous proposons une nouvelle stratégie thérapeutique. En conclusion, nous démontrons que c-Kit est un membre de la famille des récepteurs à dépendance, présentant une activité pro-apoptotique, et pouvant être utilisé comme un outil alternatif dans le cadre d'un traitement contre le cancer.

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# I. Introduction

# 1. Apoptosis and Dependence Receptor

## 1.1 Cell death

Cell death is a diverse and complex group of processes occurring in a number of forms that is ultimately caused by stress to the cellular system (Stevens et al., 2011; Galluzzi et al., 2012). A cell should be considered dead when it meets at least one of the following molecular or morphological criteria : (1) the cell has lost the integrity of its plasma membrane; (2) the cell, including its nucleus, has undergone complete fragmentation into discrete bodies (which are frequently referred to as apoptotic bodies); and/or (3) its corpse (or its fragments) has been engulfed by an adjacent cell in vivo (Kroemer et al., 2009).

In general, there are three major types of cell death, defined in large part by the appearance of the dying cell: apoptosis (also known as type I cell death), autophagic cell death (type II) and necrosis (type III) (Galluzzi et al. 2007). Apoptosis is characterized by cell shrinkage, membrane blebbing, and condensation of the chromatin (pyknosis) (Kerr et al., 1972). Autophagic cell death is characterized by the appearance of large intracellular vesicles and engagement of the autophagy machinery (Kroemer and Levine, 2008). Necrosis is characterized by cell swelling and plasma membrane rupture, and a loss of organellar structure without chromatin condensation. (Yuan and Kroemer, 2010). There are additional forms of cell death, including mitotic catastrophe (Castedo et al., 2004), ferroptosis (Dixon et al., 2012), and others.

Since we will study c-Kit as a candidate of dependence receptors, which mostly induce apoptosis in the absent of their ligands, the following introduction chapters will focus on specific programmed cell death form, that is apoptosis.

## 1.2 Apoptosis

Apoptosis is an important kind of “programmed” cell death, involving the genetically determined elimination of cells. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. On the other hand, it also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Apoptosis is an active procedure that is activated by various extra cellular lesions (hormonally active various agents, ionized radiation and other agents including chemotherapy) or by genetic

factors and it controls cellular death *via* a mechanism programmed by the cell itself (Searle, 1982).

After signal is received for apoptosis, many biochemical and morphological changes can be observed in the cell. At first, cell starts to get smaller and more condensed, the cellular skeleton is disintegrated and cellular membrane is dissolved. Nuclear DNA is divided into many pieces. The disruption of cytoplasmic skeleton of the cell leads to apoptosis. Cytoplasm starts to shrink and get smaller. Subsequently, nucleus is divided into separate fragments. Nucleus is the focus of events in apoptosis (Searle, 1982). Cell continues to shrink and become smaller and is divided into small vesicular parts surrounded by membrane and that can be recognized by macrophages. Apoptotic bodies containing cytoplasm, intact organelles and nucleus fragments (in some) can be formed (Majno and Joris, 1995). Eventually, cell dies, plasma membranes are disintegrated and organelles are observed. The chain of apoptotic events ranging from the separation of protoplasm from membrane to the formation of apoptotic body lasts a few minutes. However, phagocytosis of apoptotic cells may last 12-18 hours (Eguchi, 2001).

Differently from necrosis, in an organism there is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells, because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy dependent cascade of molecular events. To date, research indicates that there are two main apoptotic pathways: the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway). However, there is also evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002).

### **1.2.1 Extrinsic apoptotic pathway**

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi and

Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF- $\alpha$ /TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al., 1997; Ashkenazi et al., 1998; Peter and Kramer, 1998; Suliman et al., 2001; Rubio-Moscardo et al., 2005).

The sequence of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF- $\alpha$ /TNFR1 models. In these models, the initiation of the death signal requires the death ligand binding to the receptor. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP (Hsu et al., 1995; Grimm et al., 1996; Wajant, 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (Kischkel et al., 1995). Active caspase-8 is able to cleave and active caspase-3, leading to the execution phase of apoptosis.

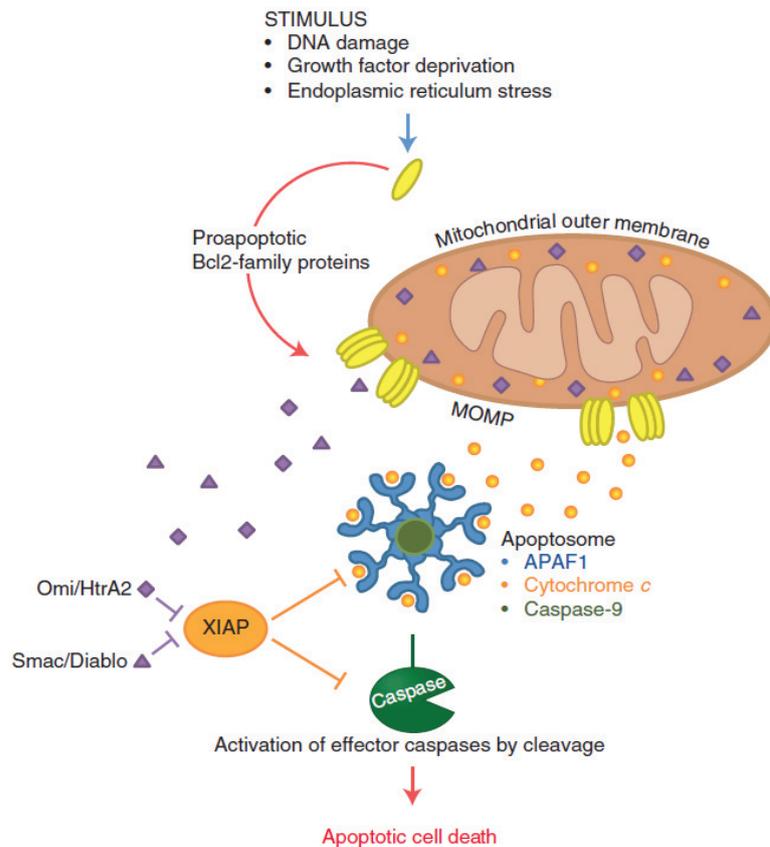
### **1.2.2 Intrinsic apoptotic pathway**

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals acting directly on targets within the cell. Those signaling pathways are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produces intracellular signals that may act in either a positive or a negative way. Negative signals involve the absence of certain growth factors, hormones and cytokines, which can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive way includes, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, genotoxic stress and free radicals (Elmore et al., 2007).

All of these stimuli cause changes in the mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai et al., 1998; Du et al., 2000; Loo et al., 2002; Garrido

et al., 2005). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming the so-called "apoptosome" (Chinnaiyan, 1999; Hill et al., 2004). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Active caspase-9 trigger further caspase-3 activation, bringing cells to execution phase of apoptosis. Factor like X-linked inhibitor of apoptosis (XIAP) is able to inhibit the activity of apoptosome, while Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting XIAP (van Loo et al., 2002a; Schimmer, 2004) (Figure 2).

The members of Bcl-2 family of proteins play a central role in the control and regulation of these apoptotic mitochondrial events (Cory and Adams, 2002), while tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins (the exact mechanisms have not yet been completely elucidated) (Schuler and Green, 2001). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. Various study has already proven that the main mechanism of action of the Bcl-2 family of proteins is the regulation of mitochondrial membrane permeability. Once the pro-apoptotic Bcl-2 family members induced MOMP, cytochrome c and other components will be released to cytoplasm and further apoptosis will be triggered (Figure 1.1).



**Figure 1.1** Intrinsic apoptotic pathway. In response to various cellular stresses, pro-apoptotic members of the Bcl-2 family induce mitochondrial outer membrane permeabilization (MOMP), allowing the release of proapoptotic factors into the cytosol that are normally sequestered in the inter-membrane space of the mitochondria (including cytochrome c, Smac, and Omi). In the cytosol, cytochrome c binds to APAF1 and triggers its oligomerization. Caspase-9 is then recruited and activated by this platform, known as the apoptosome. Catalytically active caspase-9 cleaves and activates the executioner caspases-3 and -7. Upon release in the cytosol, Smac and Omi bind to and inhibit the caspase inhibitor X-linked inhibitor of apoptosis (XIAP). In this way, they relieve XIAP inhibition of caspase-9, caspase-3, and caspase-7, and potentiate overall caspase activation by the apoptosome (Green et al., 2015).

### 1.2.3 Execution Pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, which is considered as the final step of apoptosis. This phase of apoptosis begins with the activation of the execution caspases, which then activate both cytoplasmic endonuclease (such as Mammalian endonuclease G (endo G)) that degrades nuclear material and proteases that degrade the nuclear and cytoskeletal proteins (Li et al., 2001). Caspase-3,

caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates including cytokeratins, Poly (ADP-ribose) polymerase (PARP), the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001).

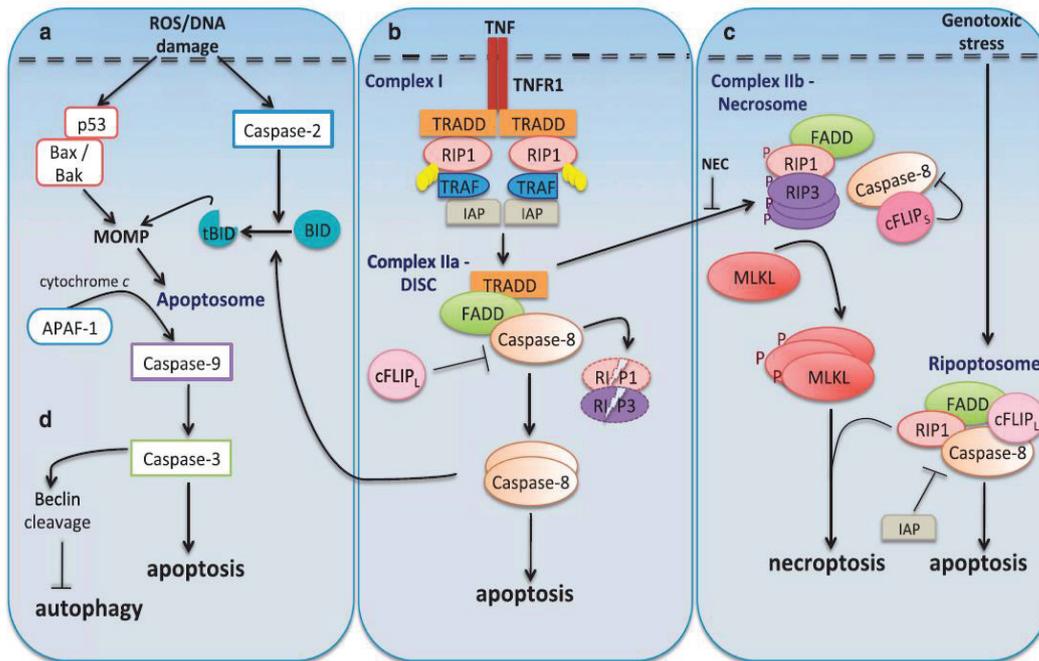
Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. The appearance of phosphatidylserine on the outer leaflet of apoptotic cells then facilitates noninflammatory phagocytic recognition, allowing for their early uptake and disposal (Fadok et al., 2001). This process of early and efficient uptake with no release of cellular constituents, results in essentially no inflammatory response.

#### **1.2.4 Caspase**

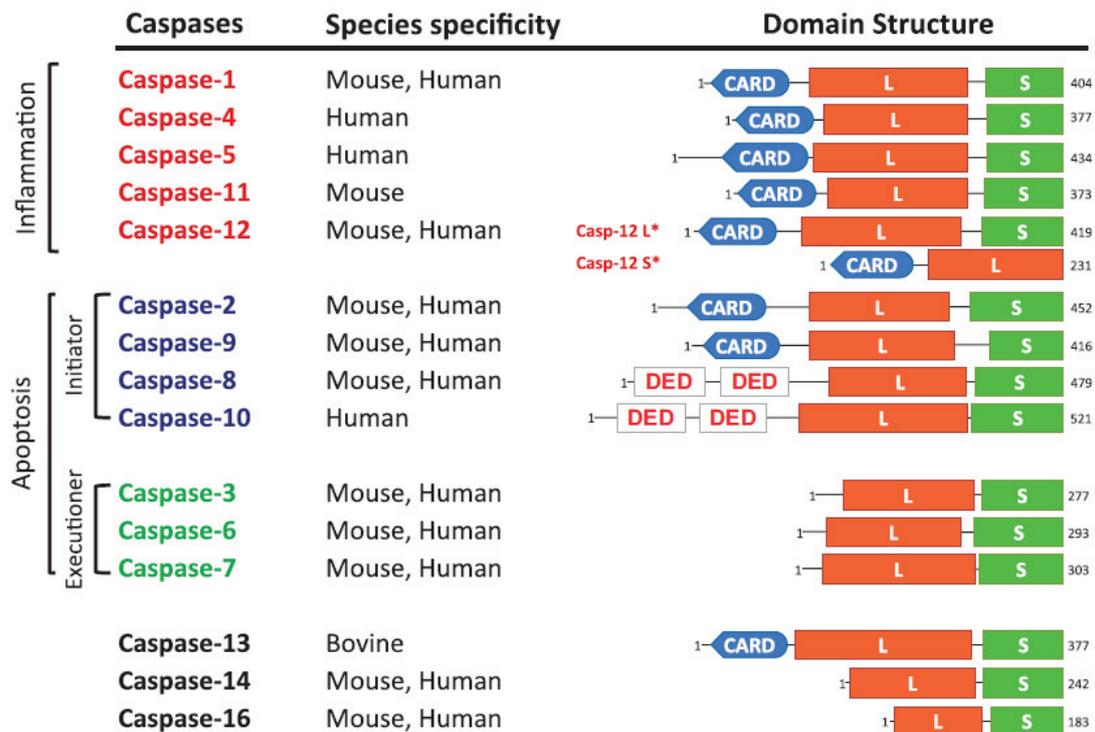
Caspases are cysteiny aspartate proteinases (cysteine proteases that cleave their substrates following an Asp residue). Almost all healthy cells contain several caspases as inactive precursors, often called caspase zymogens. Although caspase zymogens contain low level of catalytic activity, they are kept in check by a variety of regulatory molecules. Upon receiving an apoptotic signal, the caspase zymogens undergo proteolytic processing to generate two subunits that comprise the active enzyme (Fuentes-Prior et al., 2004, Degterev et al., 2003). The structural studies predict that the mature caspases are heterotetramers, composed of two heterodimers derived from two precursor molecules. In addition to the regions that give rise to the two subunits, procaspases contain N-terminal prodomains of varying lengths. Based on the length of the prodomain, caspases can be divided into two groups: those that have a relatively long prodomain, and those containing a short prodomain. The long prodomains in many caspases consist of specific protein–protein interaction domains that play a crucial role in caspase activation. These domains mediate recruitment of the procaspase molecules to specific death signaling complexes, leading to their autocatalytic activation, which is often called “proximity-induced” activation (Fuentes-Prior et al., 2004, Degterev et al., 2003). However, the detail of this mechanism is still in debate. The caspases that get activated *via* recruitment to signaling complexes known as the initiator caspases, as they initiate the caspase pathway and provide a link between cell signaling and apoptotic execution. The main initiator caspases are caspase-2, -8, -9 and -10 in mammals and Drosophila Nedd2-like caspase (DRONC) in Drosophila.

These caspases contain either a caspase recruitment domain (CARD), as in caspase-2, -9 and DRONC, or a pair of death effector domains (DEDs), as in caspase-8 and -10. These domains bind similar motifs in adaptor proteins. On the other hand, the caspases lacking a long prodomain lack the ability to self-activate and require cleavage by activated initiator caspases. Given that most of the cellular substrates are cleaved by these downstream caspases, these caspases are often called the effector caspases. The key effector caspases in mammals include caspases-3, -6 and -7, and in *Drosophila* Death related ICE-like caspase (DRICE) (Figure 1.2).

The role of these enzymes in programmed cell death was first identified in 1993, with their functions in apoptosis well characterized. Activation of caspases ensures that the cellular components are degraded in a controlled manner, carrying out cell death with minimal effect on surrounding tissues. More recently, it has become evident that the role of many caspases is not limited to apoptosis and that they function in other modes of cell death (necrosis, autophagy and pyroptosis). Furthermore, several other non-cell death functions have emerged. There are other identified roles of caspases such as cell proliferation, tumor suppression, cell differentiation, neural development and axon guidance and ageing (Shalini et al., 2015). Based on their function, mammalian caspase-2, -3, -7, -8, -9 and -10 are apoptotic caspases, whereas caspase-1, -4, -5, and 11 are considered as “inflammatory caspases” (even they may have other functions as well) (Galluzzi et al., 2016) (Figure 1.3).



**Figure 1.2** Caspases in cell death pathways. Caspases have been implicated in apoptosis, necroptosis and autophagy. Apoptosis can proceed in two ways: intrinsic or extrinsic. (a) Intrinsic apoptosis is initiated by a death-inducing stimulus, which causes activation of p53 and pro-apoptotic Bcl-2 proteins such as Bax and Bak. This leads to MOMP and activation of a death-inducing platform called the apoptosome. Following this, caspase-9 is cleaved and activates other effector caspases such as caspase-3. Alternatively, caspase-2 gets activated following DNA damage and causes MOMP by cleaving Bid. (b) Extrinsic or receptor-mediated apoptosis involves complex formation by death-domain-containing proteins (called DISC), activation of caspase-8, which initiates apoptosis by directly activating effector caspases. Caspase-8 also cleaves Bid and this causes cytochrome c release, further amplifying apoptosis via the mitochondrial/intrinsic pathway. Depending on ligands (TNF and Fas) different complexes are formed. TNF stimulates TNFR1 that recruits TRADD, RIP1, TRAFs and IAPs into a complex called TRADD-dependent complex I. Subsequent removal of polyubiquitin on RIP1, dissociates this complex and allows it to interact with TRADD, procaspase-8, FADD and cFLIP as complex IIa/DISC. This results in caspase-8 activation, leading to apoptosis. (c) In certain conditions when caspase activity is blocked, RIP1, FADD and the FLIPL–caspase-8 heterodimer, form a large multiprotein complex called the 'ripiptosome' following genotoxic stress. If caspase activity is lost, RIP1 and RIP3 are stabilized and associate in microfilament-like complexes called necrosomes (complex IIb). Recruitment of MLKL complex at the plasma membrane occurs, leading to pore formation and necroptotic cell death. Alternatively, RIPK1 and RIPK3 are inactivated by active caspase-8 and apoptosis occurs. Necroptosis can also occur following stimulation of TNF receptor or activation of Toll-like receptors (TLRs). (d) Some caspases such as caspase-3, mediate cleavage of crucial autophagic proteins and thus indirectly control autophagy. FADD, FAS-associated death domain; TNFR1, TNF receptor 1; TRADD, TNF receptor-associated death domain; cIAP1, cellular inhibitor of apoptosis protein 1; RIP1, receptor-interacting protein 1; MLKL, mixed lineage kinase domain-like; cFLIP, cellular FLICE-like inhibitory proteins; MOMP, mitochondrial outer membrane permeabilization (Shalini et al., 2015)



**Figure 1.3** Domain structure and functional classification of placental mammalian caspases. Caspase-1, -4, -5, -11 and -12 are considered as inflammatory caspases. Apoptotic caspase-2, -8, -9 and -10 are initiators, while caspase-3, -6 and -7 are key executioner caspases. CARD, caspase recruitment domain; DED, death effector domain; L, large subunit; S, small subunit; S\*, short form; L\*, long form (Shalini et al., 2015)

### 1.2.5 Apoptosis regulation in cancer

Cancer can be viewed as the result of a succession of genetic changes during which a normal cell is transformed into a malignant one. Evasion of cell death is one of the essential changes in a cell that cause this malignant transformation (Hanahan et al., 2000). As early as the 1970s, elimination of potentially malignant cells has been linked to inhibit hyperplasia and tumor progression (Kerr et al., 1972). Hence, apoptosis inhibition or resistance plays a vital role in carcinogenesis. There are many ways a malignant cell can acquire reduction in apoptosis or apoptosis resistance. Here, the major ways will be discussed as following:

## **1) BCL-2 family protein regulation**

As mentioned, the Bcl-2 family of proteins is comprised of pro-apoptotic and anti-apoptotic proteins that play a pivotal role in the regulation of apoptosis, especially via the intrinsic pathway through mitochondrial (Gross et al., 1999). When there is disruption in the balance of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, the result is dysregulated apoptosis in the affected cells. This can be due to an overexpression of one or more anti-apoptotic proteins or an underexpression of one or more pro-apoptotic proteins or a combination of both. For example, Raffo et al showed that the overexpression of Bcl-2 protected prostate cancer cells from apoptosis (Raffo et al., 1995), while Fulda et al reported Bcl-2 overexpression led to inhibition of TRAIL-induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cells (Fulda et al., 2002). Overexpression of Bcl-XL has also been reported to confer a multi-drug resistance phenotype in tumor cells and prevent them from undergoing apoptosis (Minn et al., 1995).

## **2) Inactivation of p53**

The p53 protein, also called tumor protein 53 (or TP 53), is one of the best known tumor suppressor proteins encoded by the tumor suppressor gene TP53 located at the short arm of chromosome 17 (17p13.1). It is named after its molecular weights, i.e., 53 kDa (Levine et al., 1991). p53 is not only involved in the induction of apoptosis but it is also a key player in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence (Oren et al., 1999) and is called the “guardian of the genome” (Lane, 1992).

Defects in the p53 tumor suppressor gene have been linked to more than 50% of human cancers (Bai et al, 2006). It has been reported that some target genes of p53 involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma cells, leading to abnormal activity of p53 and contributing to the proliferation of these cells (Avery-Kiejda et al., 2011). In a mouse model with an N-terminal deletion mutant of p53 ( $\Delta 122p53$ ) that corresponds to  $\Delta 133p53$  in humans, these mice had a different and more aggressive tumor spectrum, a marked proliferative advantage on cells, reduced apoptosis and a profound proinflammatory phenotype (Slatter et al., 2010).

### **3) Upregulation of Inhibitor of apoptosis proteins (IAPs)**

The Inhibitor of apoptosis proteins (IAPs) are a group of structurally and functionally similar proteins that regulate apoptosis, cytokinesis and signal transduction. To date, eight IAPs have been identified, namely, NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/MLIAP (BIRC7) and IAP-like protein 2 (BIRC8) (Vucic et al., 2007).

Dysregulated IAP expression has been reported in many cancers. For example, Lopes et al demonstrated abnormal expression of the IAP family in pancreatic cancer cells and that this abnormal expression was also responsible for resistance to chemotherapy. On the other hand, Livin was demonstrated to be highly expressed in melanoma and lymphoma (Vucic et al., 2000; Ashhab et al., 2001) while Apollon, was found to be upregulated in gliomas and was responsible for cisplatin and camptothecin resistance (Chen et al., 1999).

### **4) Reduced caspase activity**

As discussed in the previous chapter, caspases remain one of the most important players in the initiation and execution of apoptosis. It is therefore reasonable to believe that low levels of caspases or impairment in caspase function may lead to a decrease in apoptosis and promotion of carcinogenesis. In one study, downregulation of caspase-9 was found to be a frequent event in patients with stage II colorectal cancer and correlates with poor clinical outcome (Shen et al., 2010). In another study, researchers observed that caspases-3 mRNA levels in commercially available total RNA samples from breast, ovarian, and cervical tumors were either undetectable (breast and cervical) or substantially decreased (ovarian) (Devarajan et al., 2002).

### **5) Impaired death receptor signaling**

Death receptors and ligands of the death receptors are key players in the extrinsic pathway of apoptosis. Several abnormalities in the death signaling pathways that can lead to evasion of the extrinsic pathway of apoptosis have been identified. Such abnormalities include downregulation of the receptor and impairment of receptor function, as well as a reduced level in the death signals, all of which contribute to impaired signaling and hence a reduction of apoptosis. For instance, downregulation of receptor surface expression has been indicated in some studies as a mechanism of acquired drug resistance. A reduced

expression of CD95 was found to play a role in treatment-resistant leukemia (Friesen et al., 1997) or neuroblastoma (Fulda et al., 1998) cells. Reduced membrane expression of death receptors and abnormal expression of decoy receptors, which bind to death ligand without containing death domains, have also been reported to play a role in the evasion of the death signaling pathways in various cancers (Fulda, 2010).

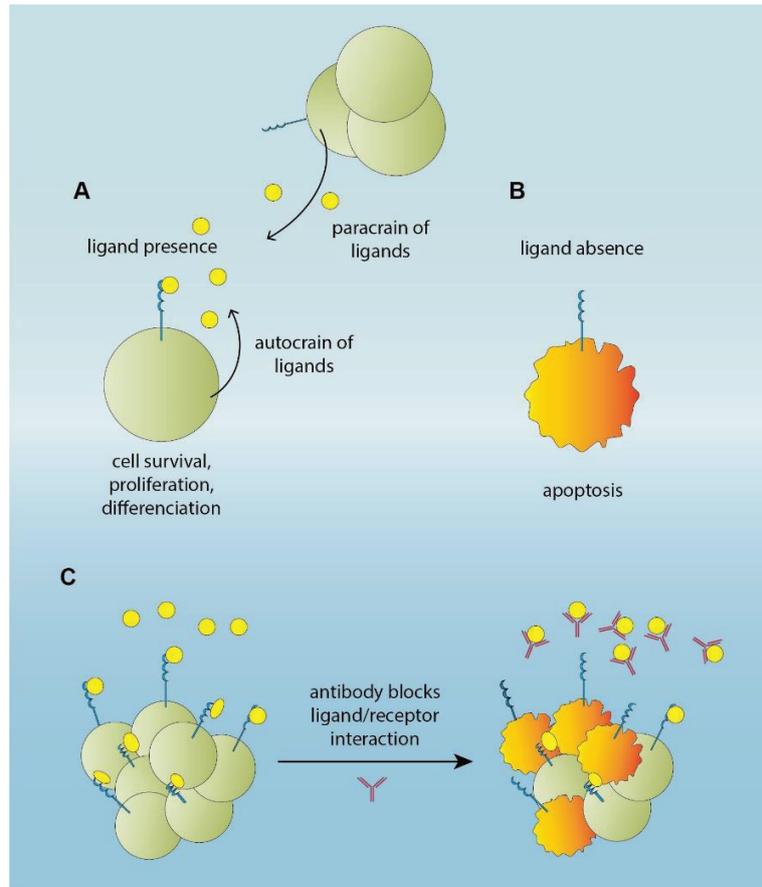
### **1.3 The dependence receptor family**

The signaling pathway network controlling cell death and survive has always been complex, with decades of study focusing on it. On the membrane of cells, a variety of receptors offer positive signaling supporting cell survival in the presence of their corresponding ligands. Thus, it has generally been assumed that cell death induced by withdrawal of ligands is due to the loss of the associated positive survival signals. Although such survival signals are clearly very important, data obtained from the past 20 years argue for a complementary and novel form of signal transduction that actively induces cell death following ligands withdrawal. This "negative signal transduction" is mediated by specific "dependence receptors" (DR) that induces apoptosis in the absence of the corresponding ligands, while this cell death signaling is blocked in the presence of the corresponding ligands.

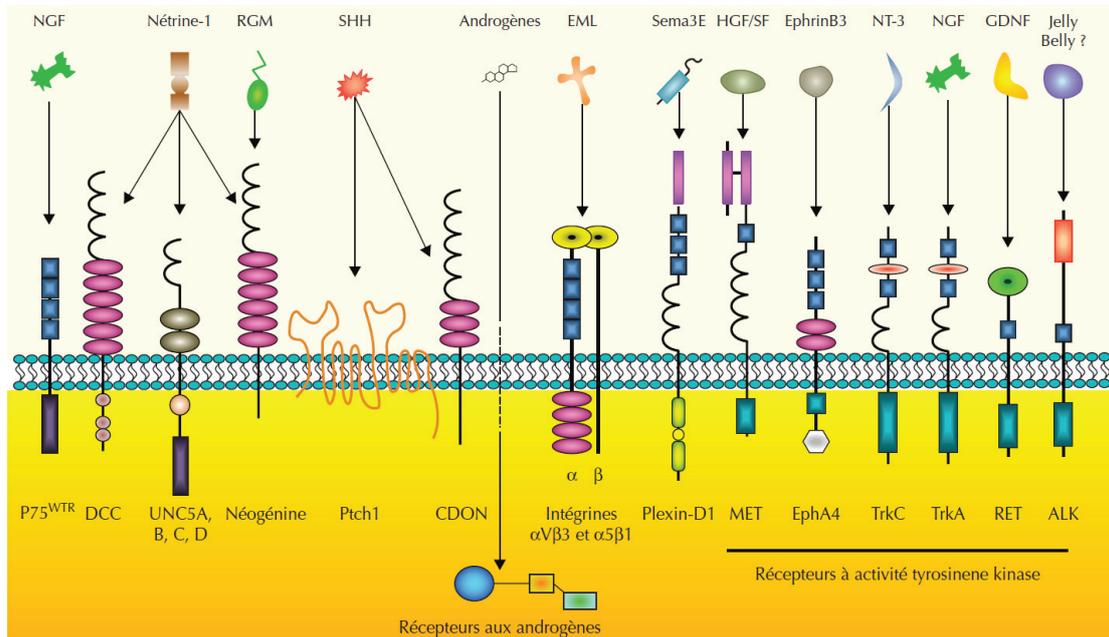
First described in 1993, these receptors play fundamental roles in processes as disparate as development, oncogenesis, and neurodegeneration (Rabizadeh et al., 2003; Goldschneider et al., 2010). The classic view of transmembrane receptors is that they are activated by binding their respective ligands, while stay relatively inactive when unbound. However, dependence receptors mediate apoptosis in the absence of their ligands, but support cell survival after ligand binding (Goldschneider et al., 2010). Thus, the presence of these receptors creates a state of dependence or addiction for the affected cells on the trophic ligands (Mehlen and Bredesen, 2004). Furthermore, dependence receptors may also mediate sub-apoptotic events, in which caspases may be activated but cell death does not ensue, depending on cell type and developmental state (Mehlen and Thibert, 2004). These receptors are thus bifunctional. In the presence of their ligands, they transduce a "positive" signal, which includes activation of various signaling pathways, such as the mitogen-activated protein kinase (MAPK)– or phosphoinositide 3-kinase (PI3K)–dependent pathways. However, in the absence of their ligands, they are not inactive but

rather trigger a “negative” signal, which leads to programmed cell death or sub-apoptotic events, such as process retraction or cellular atrophy (Goldschneider et al., 2010; Bredesen et al., 2005) (Figure 1.4 A,B). These receptors may integrate biochemical environmental information from disparate sources—such as trophic factors, hormones, and extracellular matrix components— then lead to critical cellular decisions, such as survival or programmed cell death, synaptic maintenance or synaptosis, and process extension or retraction. On the other hands, by blocking the ligand-receptor interaction (for example, by using an antibody targeting the ligand), we are able to induce cell death in cells expressing dependence receptors, offering a novel therapeutic strategy targeting concerning diseases like cancer (Figure 1.4 C).

To date, more than 20 receptors has been identified as dependence receptors (Gibert and Mehlen, 2015), sharing the ability to induce cell death in the absence of their respective ligand. This includes but not limited to the netrin-1 receptors DCC and UNC5H (Mehlen et al., 1998; Llambi et al., 2001), Sonic Hedgehog receptors Patched and CDON (Thibert et al., 2003; Delloye-Bourgeois et al., 2013) or more recently Kremen1 (Causeret et al., 2016) or Notch3 (Lin et al., 2017) (Figure 1.5).



**Figure 1.4 A,B** In the physiologic situation, dependence receptors share the property of inducing two types of signaling according to the presence or the absence of their respective ligands. In the presence of ligands (A), which are from autocrine or paracrine production, dependence receptors transduce a positive signal known to promote cell survival, migration, and/or proliferation. In the absence of ligands (B), dependence receptors can induce an apoptotic signaling, in most cases associated with proteolytic cleavage of the intracellular domain of the receptor by caspases. **C.** Dependence receptor induced cell death as therapeutic target in cancer. Monoclonal antibodies (such as anti-netrin-1) are able to block ligand– receptor interaction and trigger cell death in tumors through the pro-apoptotic activity of dependence receptors.



**Figure 1.5** Dependence receptors family members. More than 20 receptors have been discovered as dependence receptors and more candidates are currently under research (Delcros and Mehlen, 2013).

#### 1.4 Dependence receptors are caspase substrates

The molecular hallmark of programmed cell death (apoptosis) is the activation of caspases (Thornberry and Lazebnik, 1998). During apoptosis, caspases can cleave a wide range of substrates, thereby inactivating survival and activating proapoptotic mechanisms. Interestingly, most of the dependence receptors are cleaved by caspases. The importance of the caspase cleavage on dependence receptors have been proven by the facts that mutation of the cleavage site abolishes cell death induction by dependence receptors. DCC, neogenin, Ptc, ALK, EphA4 are cleaved once, roughly in the middle of their intracellular domain (Mehlen et al., 1998; Thibert et al., 2003; Matsunaga et al., 2004; Murali et al., 2006; Furne et al., 2009), while the cleavage site of UNC5 receptors is very close to the plasma membrane (Liambi et al., 2001). On the contrary, RET, Trkc and MET receptors have two cleavage sites (Bordeaux et al., 2000; Foveau et al., 2007; Tauszig-Delamasure et al., 2007). The cases of p75<sup>NTR</sup> and integrins are less clear, as there is no solid evidence that caspase cleavage is needed for their proapoptotic effect. Interestingly, caspase cleavage sites of dependence receptors seem to be conserved in

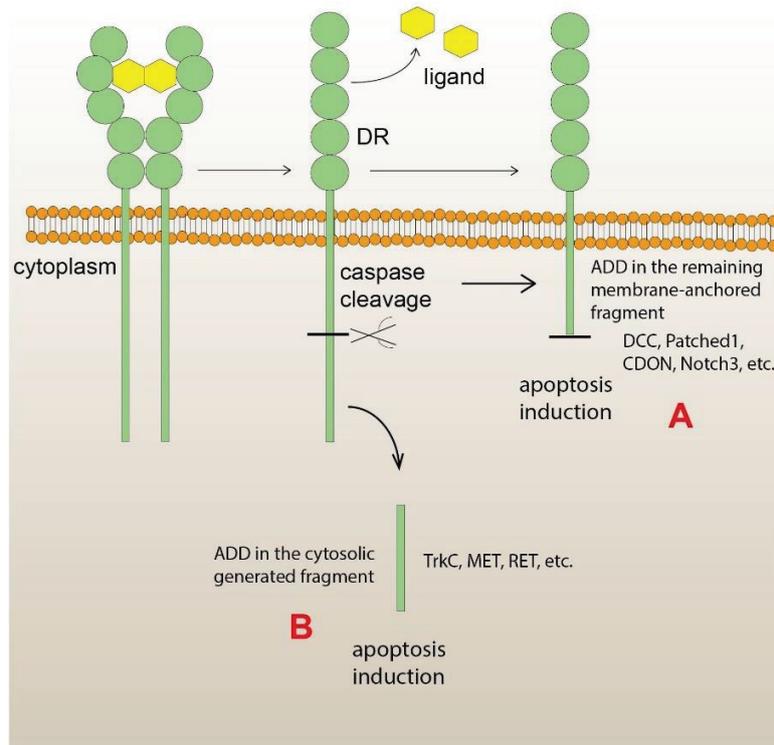
mammals, variably in other vertebrates but never in orthologs in lower organisms, such as nematode or *Drosophila* (Table 1.1) (Mehlen and Thibert, 2004). These findings may suggest that the appearance of dependence receptors as a caspase substrate, and therefore the mediation of the dependence state, is a relatively late event in the evolution of these proteins.

	<i>Homo sapiens</i>	<i>Pan troglodytes</i>	<i>Bos taurus</i>	<i>Canis familiaris</i>	<i>Mus musculus</i>	<i>Rattus norvegicus</i>	<i>Gallus gallus</i>	<i>Xenopus tropicalis</i>	<i>Xenopus laevis</i>	<i>Danio rerio</i>
DCC	LSVD	LSVD	NA	LSVD	LSVD	LSVD	NA	NA	LTVD	No
UNC5B	DITD	DITD	DITD	DITD	DITD	DITD	DITD	NA	DITD	EITD
Neogenin	CCTD	CCTD	CCTD	CCTD	CCTD	CCTD	PCAD?	GPED?	NA	CTTD
Ptc	PETD	PETD	PETD	PETD	PETD	PETD	NEDD?	HEND?	HEND?	No
RET	VSVD	VSVD	VSVD	VSVD	VPVD	VSVD	VSVD	NA	MSVD	VAID
TrkC	DYLD	DYLD	DYLD	DYLD	DYLD	DYLD	DYLD	NA	DYLD	DYLD
	SSLD	SSLD	SSLD	SSLD	SSLD	SSLD	SSLD	NA	NA	NA
EphA4	ILVD	ILVD	ILVD	ILVD	ILVD	ILVD	ILVD	NA	NA	NA
	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEED
ALK	DELD	DELD	DELD	DELD	DELD	DELD	DELD	DEMD	NA	DELD
MET	ESVD	ESVD	ESVD	ESVD	ESVD	ESVD	ESVD	ESVD	ESVD	ESVD
	DNAD	DNAD	No	DNID	DNID	DNID	DNTD	No	No	SNLD?

**Table 1.1** Conservation of caspase cleavage sites of dependence receptor among species (Goldschneider and Mehlen, 2010).

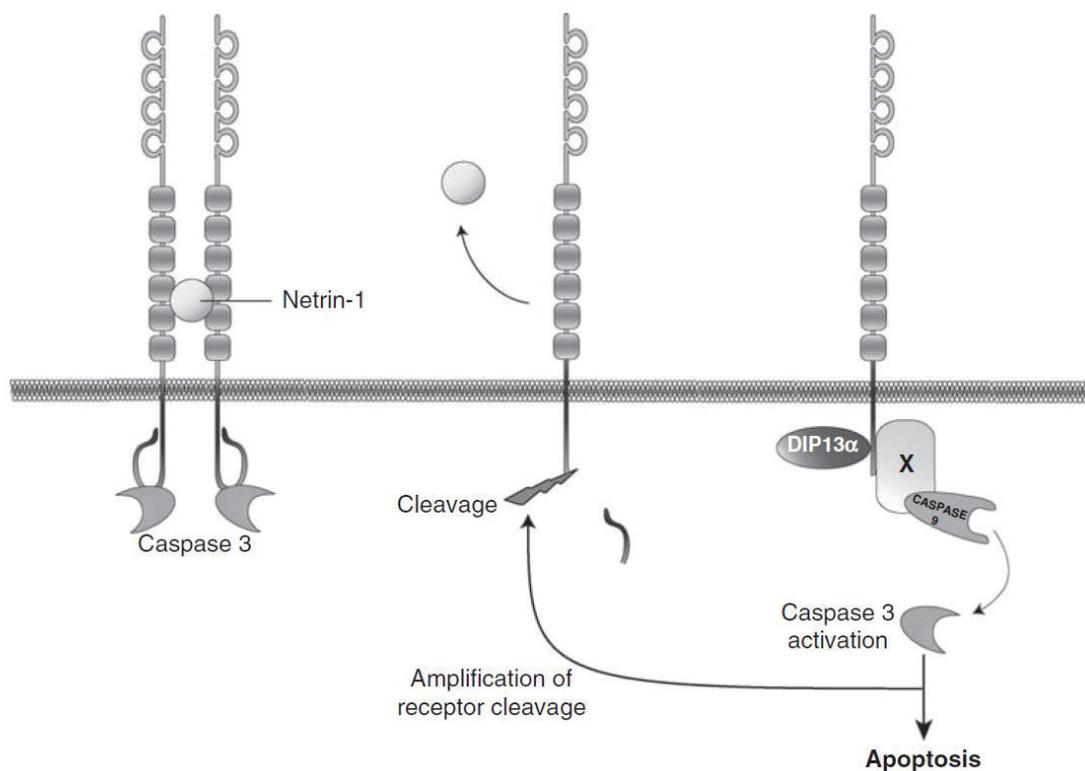
## 1.5 Mechanism of dependence receptors pro-apoptotic activity

Dependence receptors share the property of being caspase amplifiers, which has been proven by the fact that caspase inhibitors such as zVAD-fmk is able to inhibit the apoptosis induced by dependence receptors (Mehlen and Thibert, 2004). The mechanism how these dependence receptors lead to caspase activation and amplification has been described in details for some dependence receptor family members. First, all dependence receptors contain, in their intracellular part, a domain required for apoptosis induction. This domain, the ADD (addiction/ dependence domain) (Bredesen et al., 1998), is required and often sufficient for cell death induction. Caspase cleavage, except for p75NTR and integrins, is thought to be responsible for unmasking the ADD. In some dependence receptors, ADD is in by the remaining membrane-anchored fragment of the receptor after cleavage (Figure 1.6A). However, in some others, such as UNC5H, RET, TrkC and MET receptors, it is the cytosolic generated fragment that is proapoptotic (Figure 1.6B). ADDs are usually unique regions that are not structurally related to known protein functional domains. There is not many common structure or sequences among the ADDs of different dependence receptors.



**Figure 1.6** Majority of DRs have ADDs, either in the remaining membrane-anchored fragment (A) or cytosolic generated fragment (B) of the receptor after caspase cleavage. Most DRs are cleaved by caspases when the ligands are absent, producing two (or even more) fragments: membrane-anchored fragment and cytosolic fragment. The ADD of the DRs could be in either of these fragments (ADDs in membrane-anchored fragment: DCC, Patched1, CDON, Notch3, etc; ADDs in cytosolic fragment: TrkC, MET, RET, etc). The fragment containing ADD is able to induce apoptosis.

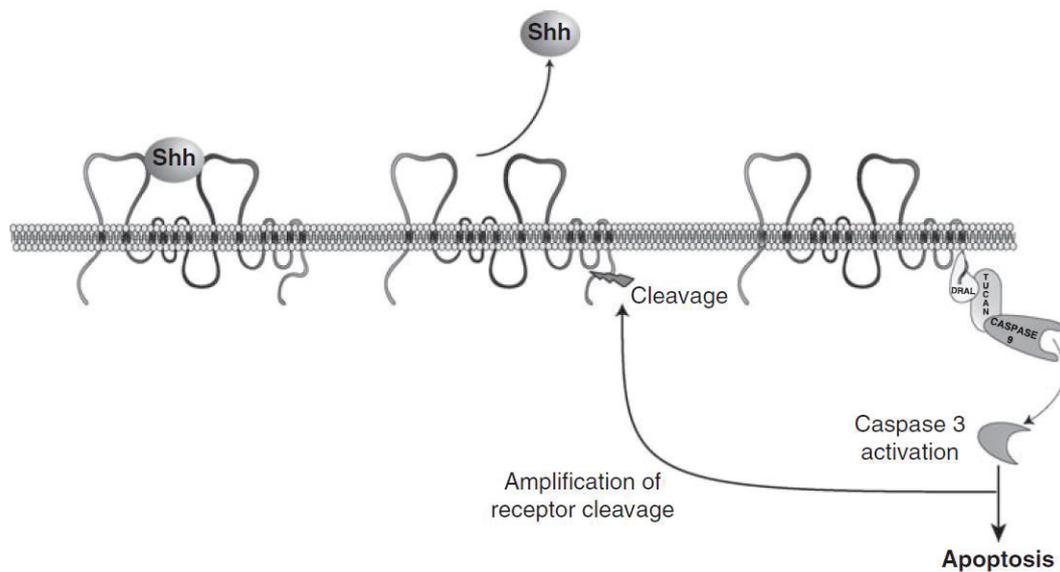
After ADD release/exposition, caspase amplification seems to be more or less direct, depending on receptors. In some cases, ADD recruits caspase-activating complexes that are different from those implicated in death receptors or in intrinsic mitochondrial classical apoptotic pathways. For example, in the absence of netrin-1, DCC recruits and activates caspase-9, thereby allowing caspase-3 activation, but this process does not require cytochrome c release and subsequent formation of an apoptosome (cytochrome c/apaf-1/caspase 9) complex, as is the case in the classic mitochondrial pathway (Forcet et al., 2001). DCC does not interact directly with caspase-9, hence it may recruit one or more adaptor proteins (Figure 1.7). One of them could be DIP13a (DCC-interacting protein 13-a), a protein identified as an interactor of DCC ADD, and shown to be important for DCC-induced cell death (Liu et al., 2002).



**Figure 1.7** Model of cell death induction by DCC. In the presence of netrin-1, DCC is dimerized and interacts with procaspase-3. Following ligand withdrawal, DCC becomes a monomer and is cleaved, possibly by bound caspase-3 or another activated protease. Cleavage leads to ADD exposure and to its direct interaction with apoptotic partners such as DIP13a, the role of which remains unclear, or to indirect interaction with caspase-9. Consequently, these interactions lead to caspase-9 activation, which in turn activates caspase-3 (Goldschneider and Mehlen, 2010).

The discovery of a caspase-activating complex recruited to a dependence receptor has been made for Patched (Ptc). In the absence of its ligand Shh, the ADD of Patch is able to interact with DRAL/FHL2 (Mille et al., 2009b). DRAL was already known to promote apoptosis through an unknown mechanism in a wide variety of cells when overexpressed (Scholl et al., 2000) and to interact with TUCAN, a CARD-containing adaptor protein for caspases-1 and -9 (Stilo et al., 2002). Mille and colleagues showed that the Ptc-DRAL association serves as a platform for recruiting TUCAN (and/or NALP1, a protein closely related to TUCAN) and caspase-9 (Figure 1.8). This then allows caspase-9 recruitment to Ptc and caspase-9 activation, further caspase-3 and downstream apoptotic pathway is

activated. The complex involving DRAL, TUCAN and caspase-9 was named “dependosome”, by analogy to other known caspase-activating complexes.

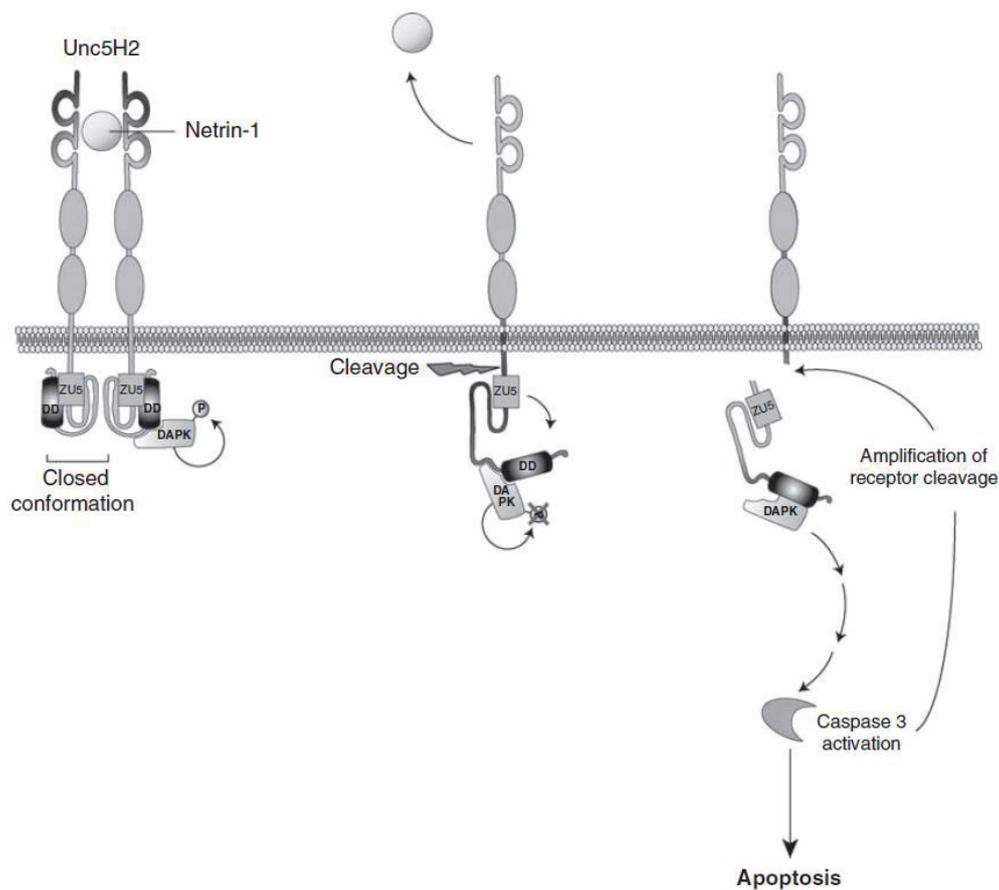


**Figure 1.8** Model of cell death induction by Ptc. Following ligand withdrawal, Ptc is cleaved by caspase (or by another activated protease), thus allowing exposure of its ADD. Through its ADD, Ptc recruits DRAL, which in turns recruits TUCAN (or NALP1) and caspase-9. Formation of this complex leads to caspase-9 activation and consequently to caspase-3 activation (Goldschneider and Mehlen, 2010).

In any case, if such a dependosome exists and is common to dependence receptors, the initiator recruited caspase is not always necessary to be caspase-9. Indeed, Stupack et al elegantly showed that integrins, as dependence receptors, trigger apoptosis through recruitment of caspase-8 (Stupack et al., 2001).

The formation of a caspase-activating complex does not seem to be the only mechanism used by dependence receptors to trigger apoptosis. As an example, the mechanism of UNC5H-induced apoptosis has been documented. Despite their structural homology, members of the UNC5H family seem to mediate their apoptotic signal by interacting preferentially with distinct partners. UNC5H2 triggers apoptosis mainly by recruiting the serine/threonine death-associated protein kinase (DAPK) (Llambi et al., 2005). UNC5H2 and DAPK bind each other in part through their respective death domain, but this is not the only way as deletion of these domains is not sufficient to abrogate their association. Llambi and colleagues proposed that, in the presence of netrin-1, DAPK is in

an inactive autophosphorylated state, and it interacts with UNC5H2 through their non-death domain-interacting regions; whereas, in the absence of netrin-1, DAPK interacts with the death domain of UNC5H2, which allows DAPK activation (Llambi et al., 2005) (Figure 1.9). This hypothesis strongly fits with the study by Wang et al. (2009), arguing that UNC5H2 can adopt a closed conformation, preventing association of the death domain with other proteins in the presence of netrin-1; while this conformation is open following withdraw of netrin-1. Downstream effectors of DAPK in UNC5H2-mediated cell death remain to be identified, but DAPK is already known to trigger cell death through p53-dependent and -independent mechanisms. Moreover, phosphorylation of the myosin light chain by DAPK leads to membrane blebbing, a hallmark of programmed cell death (Gozuacik and Kimchi, 2006).



**Figure 1.9** Model of cell death induction by UNC5H2/UNC5B. In the presence of netrin-1, UNC5H2/UNC5B are dimerized, and their intracellular domain adopts a close conformation in which ZU5 and death domains interact with each other. DAPK interacts with the closed intracellular domain,

but is in an inactive autophosphorylated state. Following ligand withdrawal, UNC5H2 receptor becomes a monomer, whereas the intracellular domain undergoes both caspase (or another protease) cleavage and opening/ dissociation of ZU5 and DD in parallel. Relation and chronology between cleavage and opening remain unclear, but these modifications should allow interaction between the DD of UNC5H and DAPK, thus leading to DAPK activation and initiation of an apoptotic program. Precisely how activated DAPK induces apoptosis remains to be determined. DD: death domain (Goldschneider and Mehlen, 2010).

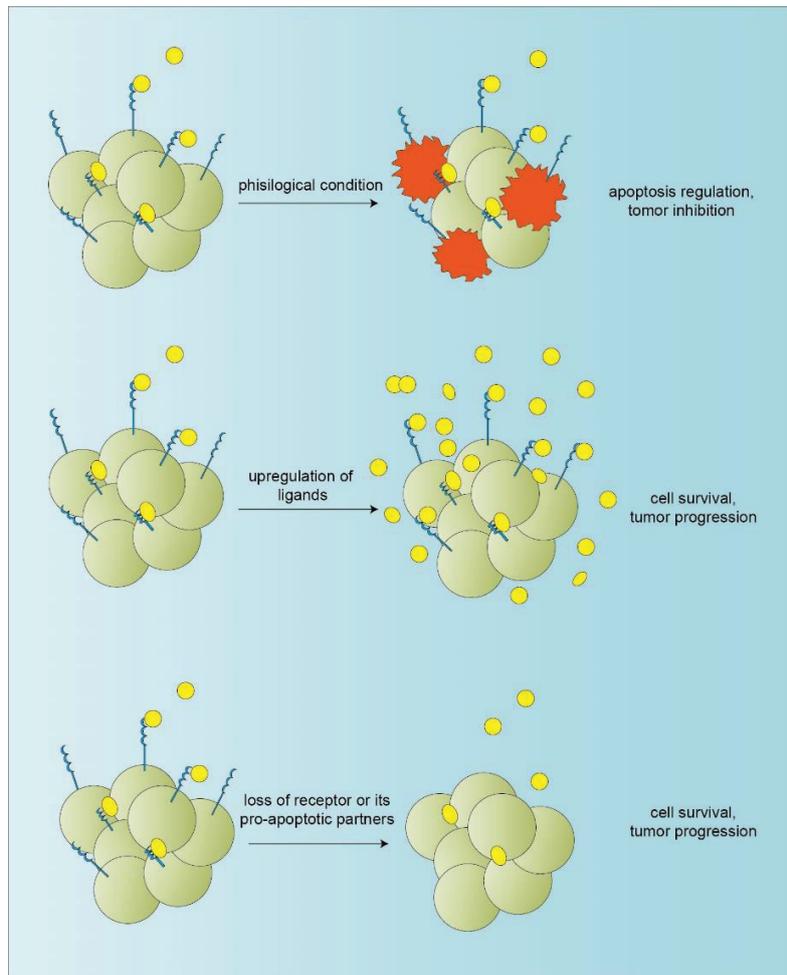
The fact that dependence receptors are at the same time caspase substrates and caspase activators/amplifiers points out a paradox. How can a receptor that requires caspase cleavage to be a proapoptotic molecule that participate in apoptosis induction? One possibility could be the initiation of dependence receptor cleavage by a non-caspase protease, which would be sufficient to generate a caspase amplification loop. Another view could be that caspases are never completely inactive, even in nonapoptotic cells, and that this residual caspase activity is sufficient to detect receptors disengaged from their ligand. Once the ligand is absent, caspase cleavage on the receptor is induced and then the following caspase pathway will amplify the caspase activity inside the cell, leading to even more caspase cleaved on dependence receptors thus again stronger caspase activity, forming a caspase cascade.

Interestingly, caspase-3 was also observed to interact with DCC, downstream of its cleavage site, in nonapoptotic conditions, that is, in the presence of netrin-1, or when the DCC caspase cleavage site is mutated (Forcet et al., 2001). It is tempting to speculate that, when dimerized in the presence of its ligand, DCC adopts a conformation that prevents its cleavage by caspases, whereas it can be efficiently cleaved as a monomer in the absence of ligand. This event results in unmasking its ADD, thus allowing caspase-9 activation and caspase-3 amplification.

## **1.6 Dependence receptors and cancer**

Since all dependence receptors have pro-apoptotic functions, they play an important role in mediating tissue homeostasis during development and tumorigenesis. Dependence receptors often act as tumor suppressors in cancer. Indeed, the expression of these receptors represents a protective mechanism that limits tumor development through apoptosis induction in tumor cells which grow or migrate beyond the regions of ligand

availability (Paradisi et al., 2010). Thus, dependence receptors have been reported to be lost or mutated in various cancer types (Goldschneider and Mehlen, 2010; Mehlen and Tauszig-Delamasure, 2014). To date, tumors have been reported to avoid the proapoptotic activity of dependence receptors via three main mechanisms: the gain of ligands, the loss or mutation of the receptor, the loss or mutations of pro-apoptotic partners of the receptors (Figure 1.10).



**Figure 1.10** Tumors develop mechanism to avoid the proapoptotic signaling of dependence receptor through different ways: upregulation of ligands, loss of dependence receptor or the proapoptotic partners, all of which prevent the pro-apoptotic activity of dependence receptor.

### **1.6.1 Netrin-1 and its receptors DCC and UNC5Hs in cancer**

Netrin-1 is a laminin-related molecule, which has been shown to act as a chemo-attractive or chemo-repulsive cue for many migrating axons and neurons. Netrin binds to two main families of type 1 transmembrane receptors: DCC (deleted in colorectal cancer) and the UNC5H, that is, UNC5 homolog receptors (UNC5H1, UNC5H2, UNC5H3, and UNC5H4, also called UNC5A, UNC5B, UNC5C, and UNC5D). Despite their roles as key mediators of nervous system development, netrin and its receptor have also been proven to be the prototype of dependence receptors.

According to the concept of dependence receptor, the mutation or the loss of the receptor would favor tumor progression. DCC, as the first identified dependence receptor, has been reported to be lost not only in colon cancer but also in prostate cancer, breast cancer, ovarian cancer, and neuroblastoma (Mehlen and Fearon, 2004). Mutation of the caspase cleavage site of DCC is sufficient to induce neoplasia in intestine and increase the tumor incidence in an adenomatous polyposis coli (APC) mutated mouse model (Castets et al., 2011). In addition, the UNC5 A-C are also found to be lost and behave as tumor suppressors in numerous cancers (Thiebault et al., 2003). Not only the loss of receptor itself, but also the loss of the pro-apoptotic partners facilitates tumor progression. DAPK1, a pro-apoptotic partner of the UNC5 family is lost in breast cancer and lung cancer via the methylation of its promoter (Grandin et al., 2016a; Grandin et al., 2016b).

On the other hand, upregulation of the ligands of dependence receptors also favors tumor progression. Along with this theory, netrin-1 has been found overexpressed in a large variety of tumor types such as breast, colon, lung, ovarian cancer, neuroblastoma, and medulloblastoma (Fitamant et al., 2008; Paradisi et al., 2008; Papanastasiou et al., 2011; Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Akino et al., 2014). Netrin-1 has also been shown associated with metastasis (Fitamant et al., 2008; Harter et al., 2014). The pro-oncogenic effect of netrin-1 expression was demonstrated by using mice overexpressing netrin-1 in the gut. These mice were more prone to develop intestinal cancer (Mazelin et al., 2004).

### **1.6.2 Neurotrophins and their dependence receptors in cancer**

Neurotrophins and their receptors are components of a signaling system which plays major and diverse roles in the nervous system of vertebrates, ranging from the control of cell death to memory retention (Chao and Ip, 2010). Each neurotrophin bind and activate

one of the three tyrosine kinase receptors identified in mammals and primarily expressed by neurons (Barbacid, 1995). Those tyrosine kinase receptors are called Trk (tropomyosin receptor kinase) A, B and C, which are highly related as they seem to have evolved from an ancestral gene by domain and exon shuffling. Except for the Trk family members, p75<sup>NTR</sup> is also a receptor of neurotrophins. It has been proven that TrkA, TrkC and p75<sup>NTR</sup> function as dependence receptors, while TrkB does not (Nikoletopoulou et al., 2010; Tauszig-Delamasure et al., 2007; Rabizadeh et al., 1993; Rabizadeh et al., 2003). Besides, the death of cells caused by TrkA and p75<sup>NTR</sup> can be prevented by the addition of nerve growth factor (NGF, ligand of TrkA and p75<sup>NTR</sup>) (Nikoletopoulou et al., 2010; Barrett et al., 1994; Rabizadeh et al., 2003), while TrkC induced cell death can be repressed by adding neurotrophin-3 (NT3, ligand of TrkC) (Tauszig-Delamasure et al., 2007).

As dependence receptors, TrkA, TrkC and p75<sup>NTR</sup> have been reported to be involved in tumor progression with the features shared by most dependence receptors. In neuroblastoma, TrkA was reported to be a good prognostic marker and its expression is negatively correlated to the up-regulation of MycN and the tumor grade (Eggert et al., 2000; Nakagawara, 1998). This is also the case for TrkC, whose expression facilitates the survival of neuroblastoma patients (Brodeur et al., 2009). It has been proposed that TrkC constrains tumor progression in neuroblastoma, and TrkC tumor suppressor activity requires p53 and transcriptional factor Hey1 (Ménard et al., 2018). According to the work of Genevois et al., the expression of TrkC is down-regulated in a large fraction of human colorectal cancers, mainly through promoter methylation. Moreover, they have shown that TrkC silencing by promoter methylation is a selective advantage for colorectal cell lines to limit tumor cell death, while reestablished TrkC expression in colorectal cancer cell lines is associated with tumor cell death and inhibition of in vitro characteristics of cell transformation, as well as in vivo tumor growth. Finally, they provided evidence that a mutation of TrkC detected in a sporadic cancer is a loss-of proapoptotic function mutation (Genevois et al., 2013). As to p75<sup>NTR</sup>, it has also been reported to inhibit tumor cell proliferation, invasion and metastasis in gastric cancer (Jin et al., 2007a; Jin et al., 2007b). In melanoma cells, apoptosis-related protein (APR)-1 causes apoptosis via the interaction with the juxtamembrane region of p75<sup>NTR</sup> (Selimovic et al., 2012).

Considering the ligands of TrkA/C and p75<sup>NTR</sup>, which are NGF and NT-3, the upregulation of these ligands should provide growth advantage for tumor cells, both through activating the kinase signaling and inhibiting the pro-apoptotic activity of the

receptors. NGF has been related to cancer progression in several tumor types (Krüttgen et al., 2006). It is secreted by breast cancer cells but not by normal breast epithelial cells (Dolle et al., 2003), and the treatment against NGF decreases breast cancer cell proliferation with an increase in apoptosis and an inhibition of tumor angiogenesis, indicating that targeting of NGF is a potential therapeutic approach in breast cancer (Adriaenssens et al., 2008). Prostate malignant epithelial cells also secrete NGF, which acts as an important autocrine factor for growth and metastasis. Interestingly, intravenous gammaglobulin (IVIg) contains natural antibodies against NGF, which inhibits growth and differentiation of the NGF-dependent prostate cancer (Warrington et al., 2011). At the same time, as the ligand of TrkC, NT-3 is overexpressed in human pancreatic cancers (Ohta et al., 1997), melanoma cells (Truzzi et al., 2008), medullar thyroid carcinoma (McGregor et al., 1999), lung cancer (Ricci et al., 2001), pancreatic cancer (Zhu et al., 1999), prostatic cancer (Weeraratna et al., 2000), ovarian carcinomas (Davidson et al., 2003) and neuroblastoma (Bouzas-Rodriguez et al., 2010).

### **1.6.3 Sonic Hedgehog and its receptors CDON and Patched in cancer**

Sonic Hedgehog (SHH) is a key secreted morphogen with multiple known functions. Canonical SHH signaling is mediated mainly *via* its interaction with the receptor Patched 1 (Ptc). Cell-adhesion molecule-related/down-regulated by Oncogenes (CDON or CDO) and Brother of CDON (BOC) have also been recently described as receptors for SHH, and hypothetical co-receptors of Ptc (Tenzen et al., 2006; Zhang et al., 2006; Izzi et al., 2011). Both Ptc (Mille et al., 2009) and CDON (Delloye-Bourgeois et al., 2013) have been proven to be members of dependence receptors, triggering apoptosis in the absence of ligand SHH. It has been reported that CDON expression is decreased in some colorectal, lung, kidney and breast cancers, and CDON is a *bona fide* tumor suppressor in colorectal cancer (Delloye-Bourgeois et al., 2013). Another report indicates that CDON expression was inversely associated with neuroblastoma aggressiveness, and re-expression of CDON in neuroblastoma cell lines led to apoptosis *in vitro* and tumor growth inhibition *in vivo* (Gibert et al., 2014). Besides, CDON expression is down-regulated by the miR181, whose expression is directly associated with neuroblastoma aggressiveness (Gibert et al., 2014). Concerning Ptc, about 30% of Ptc heterozygous mice develop medulloblastoma (Goodrich et al., 1997), indicating that Ptc possibly acts as a tumor suppressor for medulloblastoma.

On the other hand, according to dependence receptor theory, tumors may have been selected for up-regulation of SHH, which should be associated with a constitutive inactivation of CDON or Patched induced cell death. Following this hypothesis, autocrine or paracrine expression of SHH has been extensively described in different human cancers (Watkins et al., 2003; Thayer et al., 2003; Yauch et al., 2008; Scales et al., 2009; Stecca et al., 2007; Tian et al., 2009). Traditionally, this autocrine or paracrine SHH expression is viewed as a mechanism that constitutively activates Ptc-Smo signaling either in tumor cells or more generally in stromal cells (Yauch et al., 2008; Scales et al., 2009). Since both CDON and Ptc are able to induce apoptosis, the presence of SHH does not only activate the canonical signaling in stromal cells but also (or rather) blocks CDON and Ptc mediated tumor cell death.

#### **1.6.4 Semaphorin 3E and Plexin D1**

The semaphorins constitute one of the largest families of guidance molecules, which use plexin proteins as their main signal-transducing receptors. The receptor of semaphorin 3E (Sema3E) is Plexin D1, which has already been illustrated as a member of dependence receptor, triggering apoptotic cell death when dissociated from its Sema3E via an NR4A1-dependent mitochondrial apoptotic pathway (Luchino et al., 2013). At the same time, potentiating this dependence receptor pathway by using a Plexin D1 ligand trap is sufficient to reduce tumor growth and metastasis in preclinical models of breast cancer (Luchino et al., 2013).

As the ligand of Plexin D1, upregulation of Sema 3E promotes tumor cell survival, thus favoring tumor progression. In fact, expression level of Sema3E ligand appears to be positively correlated with increased metastasis in breast, ovarian, melanoma, and colon cancers and with poor patient survival in colorectal and pancreatic cancers (Luchino et al., 2013; Biankin et al., 2012; Casazza et al., 2010; Tseng et al., 2011). Plexin D1 has not yet been shown lost or mutated in any cancers. But its pro-apoptotic partner, NR4A1, is negatively associated with histologic grade in breast tumors, with reduced expression of NR4A1 in higher grade and metastatic tumors (Alexopoulou et al., 2010; Muscat et al., 2013).

#### **1.6.5 Neogenin and RGMs (repulsive guidance molecules)**

Neogenin is a homologue of DCC. The major ligands of Neogenin are the RGM family members, including RGMa, RGMb and RGMc (Matsunaga et al., 2006). Neogenin was

proposed to mediate the axon guidance function of RGMs (Rajagopalan et al., 2004), and the RGM/neogenin pair has been shown to play a role in neuronal differentiation (Matsunaga et al., 2006). Just like its homologue DCC, Neogenin has also been shown to belong to the dependence receptor family (Matsunaga et al., 2004). It has been reported as a tumor suppressor in gliomas and its down-regulation owing to promoter methylation is a selective advantage for glioma genesis, progression and recurrence (Wu et al., 2012). Another report indicates that Neogenin may play a role in mammary carcinogenesis as well as morphogenesis, and the expression may be inversely correlated with mammary carcinogenicity (Lee et al., 2005). Similar study in breast cancer also shows that neogenin expression in breast cancer tissues is inversely associated with tumor grade *in vivo* (Xing et al., 2014). Concerning RGMs, the expression of RGMb has been reported to be increased in colon cancer tissues compared to control tissues in mouse model and in human patients (Shi et al., 2015).

#### **1.6.6 MET, RET, EphA4 receptor tyrosine kinases as dependence receptors in cancer**

MET (MNNG HOS transforming gene), RET (REarranged during Transfection) and EphA4 (Ephrin Receptor A4) have been traditionally viewed only as receptor tyrosine kinases. However, similar to TrkC, study shows that MET, RET and EphA4 are also members of dependence receptors (Tulasne et al., 2004; Bordeaux et al., 2000; Furne et al., 2009).

RET has tumor suppressor activity in colon cancer and it is aberrantly methylated in 27% of colon adenomas and in 63% of colorectal cancers, correlating with RET expression silencing (Luo et al., 2013). Besides, RET mutations, which inactivate RET, have also been found in primary colorectal cancer (Luo et al., 2013). The ligands of RET, glial cell line-derived neurotrophic factor (GDNF) family members, have been reported aberrantly high expressed in glioma, possibly due to changes in promoter region methylation that causes stronger transcriptional factor binding capacity (Yu et al., 2013), or due to hyperacetylation of histone H3 lysine 9 (H3K9) at transcription factor early growth response protein 1 (Egr-1) binding sites in promoter region II of the GDNF gene (Zhang et al., 2014). Besides, GDNF family receptor (GFR) $\alpha$ 1 released by nerves enhances cancer cell perineural invasion (He et al., 2014). Transcripts encoding GDNF increase several fold following exposures to cytotoxic agents, causing secretion of GDNF in the tumor microenvironment with paracrine effects promoting prostate cancer treatment resistance (Huber et al., 2015).

As to MET, there is a report indicating that allelic loss of the c-MET was detected in both primary (13%) and metastatic sites (31%) of breast cancer (Ng et al., 1997). At the same time, an overexpression of its ligand HGF (hepatocyte growth factor receptor)/SF (scatter factor) has been notably observed in breast, gastric, colon, or lung cancers and associated with a poor prognosis (Ujiie et al., 2012). Moreover, recent studies have highlighted a key role for HGF/SF secreted from the tumor microenvironment in the development of drug resistance, especially to RAF inhibitors (Straussman et al., 2012). Even though the overexpression of HGF/SF has been generally considered only related to an upregulation of MET kinase activation, it should also repress the proapoptotic activity of MET since MET has been proven to act as a dependence receptor, thus favoring tumor progression (Tulasne et al., 2004; Lefebvre et al., 2013; Furlan et al., 2014).

EphA4 has been reported to be expressed at higher levels in lung cancer compared with non-cancer tissues and EphA4 gene expression is associated with an improved outcome in patients with resected lung adenocarcinoma, possibly by affecting cancer cell migration and invasion (Saintigny et al., 2012). Another study shows that EphA4 is reduced in breast carcinoma, which is associated with high grade, advanced TNM stage, lymph node metastasis, and poor outcome of patients (Sun et al., 2016). At the same time, the ligand of EphA4, Ephrin-B3, is highly expressed in glioblastoma, acting as a survival factor and promoting angiogenesis for tumoral cells via inhibition of EphA4-induced cell death (Royet et al., 2017).

### **1.6.7 Other dependence receptors in cancer**

#### **Kremen1**

Kremen1(Krm1) is a newly identified dependence receptor with an intrinsic pro-apoptotic activity, which can be inhibited by its ligand Dickkopf1 (Dkk1) (Causeret et al., 2016). Causeret et al. also show that in the cancer genome atlas (TCGA), various cancers fit within a pattern combining (i) high expression of Krm1 and low expression of Dkk1 in normal tissue, (ii) decreased Krm1 expression in tumors, and/or (iii) increased Dkk1 expression in tumors. Furthermore, they found Kremen1 mutations in human cancers that can affect its apoptotic activity and propose that these mutations favor abnormal cancer cells survival and thus confer them a selective advantage (Causeret et al., 2016).

### Notch3 (Neurogenic locus notch homolog protein 3)

Receptor Notch3 is a major player of tumor angiogenesis. Recently, Notch3 has been brought into the family of dependence receptor (Lin et al., 2017). Research shows that Notch3 is aberrantly expressed in the pathological tumor vascularization where Notch3 limits tumor angiogenesis through its pro-apoptotic activity (Lin et al., 2017). Jagged-1, the ligand of Notch3, has been found to be overexpressed and promoting tumor progression in breast cancer, brain cancer, cervical cancer, colorectal cancer pancreatic cancer, hepatocellular cancer, endometrial cancer, renal cancer, acute myeloid leukemia and some types of lymphoma (Li et al., 2014). Therefore, targeting more specifically the interaction between Notch3 and its ligand Jagged-1 could be advantageous allowing targeting of both the canonical Notch signaling and Notch3-induced apoptosis in endothelial cells.

#### 1.6.8 Summary of dependence receptors in cancer

In conclusion, the relation between cancer and all the dependence receptors discussed above indicates that dependence receptor plays a crucial role in many types of cancer. Both the upregulation of ligands and the loss or mutation of receptors and their pro-apoptotic partners contribute to tumor progression. Table 1.2 summarizes the implications of dependence receptors and their ligands in cancer.

ligands and dependence receptors pair		implications in cancer	
ligands	receptors	ligands in cancer	receptors in cancer
Netrin-1	DCC	1. Netrin-1 was found to be overexpressed in a large variety of tumor types such as breast, colon, lung, ovarian cancer, neuroblastoma, and medulloblastoma (Fitamant et al., 2008; Paradisi et al., 2008; Papanastasiou et al., 2011; Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Akino et al., 2014). 2. Netrin-1 is associated with metastasis (Fitamant et al., 2008; Harter et al., 2014).	1. DCC is lost in colon cancer, prostate cancer, breast cancer, ovarian cancer, and neuroblastoma (Mehlen and Fearon, 2004) 2. Mutation of the caspase cleavage site of DCC is sufficient to induce neoplasia in intestine and increase the tumor incidence in an adenomatous polyposis coli (APC) mutated mouse model (Castets et al., 2011).
	Unc5H		1. UNC5 A-C are also found to be lost and behave as tumor suppressors in numerous cancers (Thiebault et al., 2003). 2. The loss of DAPK1, a pro-apoptotic partner of UNC5 family, facilitates tumor progression in breast and lung cancer via methylation of its promoter (Grandin et al., 2016a; Grandin et al., 2016b).
NGF	p75 <sup>NTR</sup>	1. NGF has been related to cancer progression in several tumor types (Krüttgen et al., 2006). 2. NGF is secreted by breast cancer cells but not by normal breast epithelial cells (Dolle et al., 2003), and the treatment directed against NGF decreases breast cancer cell	1. p75 <sup>NTR</sup> has also been reported to inhibit tumor cell proliferation, invasion and metastasis in gastric cancer (Jin et al., 2007a; Jin et al., 2007b). 2. In melanoma cells, apoptosis-related protein (APR)-1 causes apoptosis via the interaction with the

		proliferation with a concomitant increase in apoptosis, and an inhibition of tumor angiogenesis (Adriaenssens et al., 2008).	juxtamembrane region of p75 <sup>NTR</sup> (Selimovic et al., 2012).
	TrkA	3. Prostate malignant epithelial cells secrete NGF, which acts as an important autocrine factor for growth and metastasis. Natural antibodies against NGF inhibit growth and differentiation of the NGF-dependent prostate cancer (Warrington et al., 2011).	In neuroblastoma, TrkA is reported to be a good prognostic marker and its expression is negatively correlated to the up-regulation of MycN and the tumor grade (Eggert et al., 2000; Nakagawara, 1998).
NT3	TrkC	NT-3 is overexpressed in human pancreatic cancers (Ohta et al., 1997), melanoma cells (Truzzi et al., 2008), medullar thyroid carcinoma (McGregor et al., 1999), lung (Ricci et al., 2001), pancreatic (Zhu et al., 1999), prostatic (Weeraratna et al., 2000), ovarian carcinomas (Davidson et al., 2003) and and neuroblastoma (Bouzas-Rodriguez et al., 2010).	<ol style="list-style-type: none"> <li>1.TrkC facilitates the survival of neuroblastoma patients (Brodeur et al., 2009).</li> <li>2. TrkC constrains tumor progression in neuroblastoma, and TrkC tumor suppressor activity requires p53 and transcriptional factor Hey1 (Ménard et al., 2018).</li> <li>3. The expression of TrkC is down-regulated in a large fraction of human colorectal cancers, mainly through promoter methylation, favoring tumor survival and progression. (Genevois et al., 2013)</li> <li>4. The loss-of proapoptotic function mutation of TrkC has been detected in a sporadic cancer (Genevois et al., 2013).</li> </ol>
	Patched1		About 30% of Patched1 heterozygous mice develop medulloblastoma (Goodrich et al.,1997)
SHH	CDON	Autocrine or paracrine expression of SHH has been extensively described in different human cancers (Watkins et al., 2003; Thayer et al., 2003; Yauch et al., 2008; Scales et al., 2009; Stecca et al., 2007; Tian et al., 2009).	<ol style="list-style-type: none"> <li>1. Expression is decreased in some colorectal, lung, kidney and breast cancers, and CDON is a bona fide tumor suppressor in colorectal cancer (Delloye-Bourgeois et al., 2013).</li> <li>2. CDON expression is inversely associated with neuroblastoma aggressiveness, and CDON inhibits neuroblastoma tumor growth (Gibert et al., 2014).</li> <li>3. CDON expression is regulated by the miR181, whose expression is directly associated with neuroblastoma aggressiveness (Gibert et al., 2014)</li> </ol>
Sema3E	Plexin D1	Expression levels of Sema3E ligand appear to be positively correlated with increased metastatic disease in breast, ovarian, melanoma, and colon cancers and with poor patient survival in colorectal and pancreatic cancers (Luchino et al., 2013; Biankin et al., 2012; Casazza et al., 2010; Tseng et al., 2011).	NR4A1, the pro-apoptotic partner of Plexin D1 (Luchino et al., 2013), is negatively associated with histologic grade in breast tumors, with reduced expression of NR4A1 in higher grade and metastatic tumors (Alexopoulou et al., 2010; Muscat et al., 2013).
RGMs	Neogenin	The expression of RGMb has been reported to be increased in colon cancer tissues compared to control tissues in mouse model and in human patients (Shi et al., 2015).	<ol style="list-style-type: none"> <li>1. Neogenin has been reported as a tumor suppressor in gliomas and its down-regulation owing to promoter methylation is a selective advantage for glioma genesis, progression and recurrence (Wu et al., 2012).</li> <li>2. Another report indicates that Neogenin may play a role in mammary carcinogenesis as well as morphogenesis, and the expression may be inversely correlated with mammary carcinogenicity (Lee et al., 2005). Similar study in breast cancer also shows that neogenin expression in breast cancer tissues is inversely associated with tumor grade in ex vivo (Xing et al., 2014).</li> </ol>
HGF/SF	MET	<ol style="list-style-type: none"> <li>1. An overexpression of HGF/SF has been notably observed in breast, gastric, colon, or lung cancers and associated with a poor prognosis (Ujii et al., 2012).</li> <li>2. HGF/SF secreted from the tumor microenvironment has a key role for in the development of drug resistance, especially to RAF inhibitors (Straussman et al., 2012)</li> </ol>	Allelic loss of the c-MET was detected in both primary (13%) and metastatic sites (31%) of breast cancer (Ng et al., 1997).
GDNF family ligands	RET	1.GDNF have been reported aberrantly high expressed in glioma, possibly due to changes in promoter region methylation that causes stronger transcriptional factor binding capacity (Yu et al., 2013) or due to hyperacetylation of histone H3 lysine 9 (H3K9) at transcription factor early growth response protein 1 (Egr-1) binding sites in promoter region II of the GDNF gene (Zhang et al., 2014).	<ol style="list-style-type: none"> <li>1. RET has tumor suppressor activity in colon cancer and it is aberrantly methylated in in 27% of colon adenomas and in 63% of colorectal cancers, which silences RET expression (Luo et al., 2013).</li> <li>2. RET mutations, which inactivate RET, have also been found in primary colorectal cancer (Luo et al., 2013).</li> </ol>

		<p>2. GDNF family receptor (GFR)<math>\alpha</math>1 released by nerves enhances cancer cell perineural invasion (He et al., 2014).</p> <p>3. Transcripts encoding GDNF increases several fold following exposures to cytotoxic agents, causing secretion of GDNF in the tumor microenvironment with paracrine effects promoting prostate cancer treatment resistance (Huber et al., 2015).</p>	
Ephrin-B3	EphA4	Ephrin-B3 is highly expressed in glioblastoma, acting as a survival factor and promoting angiogenesis for tumoral cells via inhibition of EphA4-induced cell death (Royet et al., 2017).	<p>1. EphA4 has been reported to be expressed at higher levels in lung cancer compared with non-cancer tissues and cell lines and EphA4 gene expression is associated with an improved outcome in patients with resected lung adenocarcinoma, possibly by affecting cancer cell migration and invasion (Saintigny et al., 2012).</p> <p>2. EphA4 is reduced in breast carcinoma, which is associated with high grade, advanced TNM stage, lymph node metastasis, and poor outcome of patients (Sun et al., 2016).</p>
Dickkopf1	Kremen1	Various cancers have low expression of Dickkopf1 in normal tissue but increased Dickkopf1 expression in tumors (Causeret et al., 2016).	<p>1. Various cancers have high expression of Kremen1 in normal tissue but decreased Kremen1 expression in tumors (Causeret et al., 2016).</p> <p>2. Kremen 1 mutations, which affects its pro-apoptotic activity have been found in human cancers, favoring abnormal cancer cell survival (Causeret et al., 2016)</p>
Jagged-1	Notch3	Jagged-1 has been found to be overexpressed and promoting tumor progression in breast cancer, brain cancer, cervical cancer, colorectal cancer pancreatic cancer, hepatocellular cancer, endometrial cancer, renal cancer, acute myeloid leukemia and some types of lymphoma (Li et al., 2014).	Notch3 is aberrantly expressed in the pathological tumor vascularization where Notch3 limits tumor angiogenesis through its pro-apoptotic activity (Lin et al., 2017).

**Table 1.2** Dependence receptors and their ligands play important roles in cancer. Both the upregulation of ligands and the loss or mutation of receptors and their pro-apoptotic partners give tumor cells a selective advantage, favoring tumor progression. DCC, deleted in colorectal cancer; UNC5H, UNC5 homolog receptors; Trk, tropomyosin receptor kinase; NGF, nerve growth factor; NT-3, neurotrophin-3; SHH, Sonic Hedgehog; Ptc, Patched 1; CDON, Cell-adhesion molecule-related/down-regulated by Oncogenes; Sema3E, semaphoring 3E; RGMs, repulsive guidance molecules; MET, MNNG HOS transforming gene; RET, REarranged during Transfection; EphA4, Ephrin Receptor A4; GDNF, glial cell line-derived neurotrophic factor; HGF, hepatocyte growth factor receptor; SF, scatter factor; Krm1, Kremen1; Dkk1, Dickkopf1; Notch3, Neurogenic locus notch homolog protein 3.

### 1.7 Dependence receptor: a new therapeutic target

Because dependence receptors induce cell death in territories where the ligand is limited, a selective advantage for a tumor is to produce the appropriate dependence receptor ligands. The overexpression of dependence receptor ligands acts in two different ways to benefit tumor cells. Firstly, the ligand may stimulate the positive signaling of these

dependence receptors that is often associated with pro-migratory, pro-proliferative effect—for example, DCC and most other dependence receptors have been shown to activate ERK/MAPK upon ligands binding (Forcet et al., 2002; Ming et al., 2002). Secondly and more important benefit is that it blocks dependence receptor-mediated cell death induction. These are some cases that several dependence receptors share the same ligand. For example, netrin-1 binds to DCC and UNC5A, B, C, D dependence receptors; Sonic hedgehog (SHH) binds to Patched (Ptc) and Cell-adhesion molecule-related/Downregulated by Oncogenes (CDON) dependence receptors. Up-regulation of one ligand could block the death induced by several dependence receptors, making this special ligand a “master controller” of cell. Besides, up-regulation of ligands including Netrin-1, Neurotrphin-3, and Semaphorin 3E (Fitamant et al., 2008; Mancino et al., 2011; Luchino et al., 2013) is also usually observed in different types of cancer and their expression could be correlated to metastasis. This makes these ligands as putative biomarkers for the prognostics and potential therapeutic targets. As a consequence, antibodies neutralizing and antagonizing specifically to the binding of ligand/receptor have been adopted as therapeutic strategy and widely developed in the past years (Broutier et al., 2016; Grandin et al., 2016b). However, increasing specificity of antibodies and identifying more biomarkers for better patient selection are still big challenges for the transition from therapeutic concept to clinical practice.

As mentioned before, netrin-1 has been found to be upregulated in various kinds of tumor and it is related to metastasis. Similarly, several *in vitro* and animal proof-of-concept studies have shown that interference of netrin-1 -receptor interaction, such as silencing netrin-1 or using a recombinant netrin-1 trap, is associated with netrin-1-expressing cancer cell death, *in vivo* tumor growth and metastasis inhibition (Fitamant et al., 2008; Paradisi et al., 2009; Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b). Interestingly, in tumor cell lines and specimens where netrin-1 is expressed at a low level, treatment with conventional drugs -- that is doxorubicin, 5-fluorouracil, paclitaxel (Taxol), and cisplatin -- is often associated with an upregulation of both netrin-1 and its receptors, probably because netrin-1 and its dependence receptors are direct transcriptional targets of p53 and related stress mediators (Paradisi et al., 2013). As a consequence, combining conventional drugs and netrin-1 interference increases tumor cell death *in vitro* and potentiates tumor growth inhibiting effect *in vivo*. This phenomenon suggests that even when tumors do not express high levels of netrin-1, a combination of conventional drugs plus drugs inhibiting netrin— receptors interaction could amplify the chemotherapy-

induced response. Recent research shows that netrin-1 may not only have a pro-oncogenic effect by blocking the death induced by DCC or UNC5H, it may also activate "pro-tumorigenic" pathways by the implication of the YAP pathway (Qi et al., 2015), thus further strengthening the rationale for moving netrin-1 interference into the clinic. A humanized monoclonal antibody disrupting netrin-1 - receptors interaction has been developed in our laboratory and is currently assessed in early clinical trial (<https://clinicaltrials.gov/ct2/show/NCT02977195>). This clinical evaluation will be crucial to validate or refute the importance of targeting dependence receptor pathways in cancer.

Interestingly, this first-in-class netrin-1 interfering compound may represent only one of a large family of drugs interfering with ligand-dependence receptor interaction. Indeed, not only netrin-1, but also ligands of other dependence receptors appear to be up-regulated in cancer. NT-3 was also shown to be overexpressed in a fraction of neuroblastoma as a potential mechanism to block TrkC-induced apoptosis both *in vitro* and *in vivo* (Bouzas-Rodriguez et al., 2010). Recently, Luchino and colleagues elegantly demonstrated that PlexinD1 acts as a novel dependence receptor, and that its ligand, Sema3E, is up-regulated in breast cancer. Using a Sema3E trap recombinant protein, they were able to demonstrate that Sema3E titration is associated with inhibition of tumor growth and metastasis in different animal models (Luchino et al., 2013).

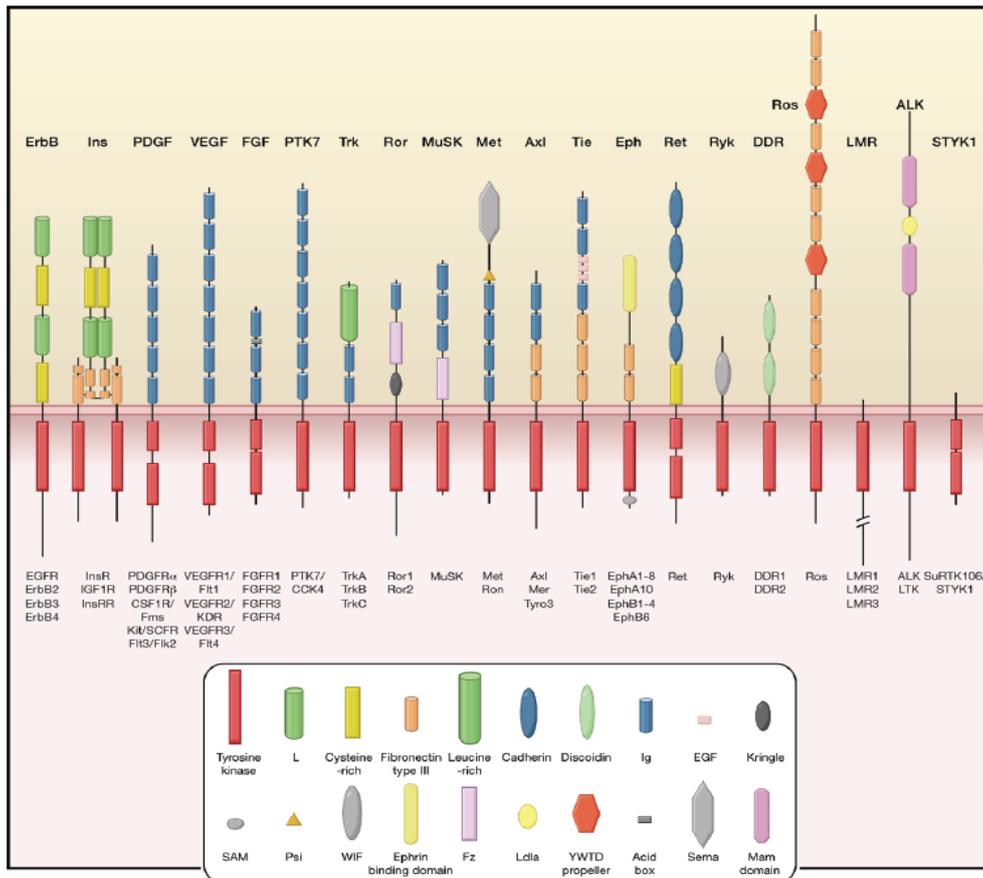
## **2. Receptor tyrosine kinase**

### **2.1 Receptor tyrosine kinase families**

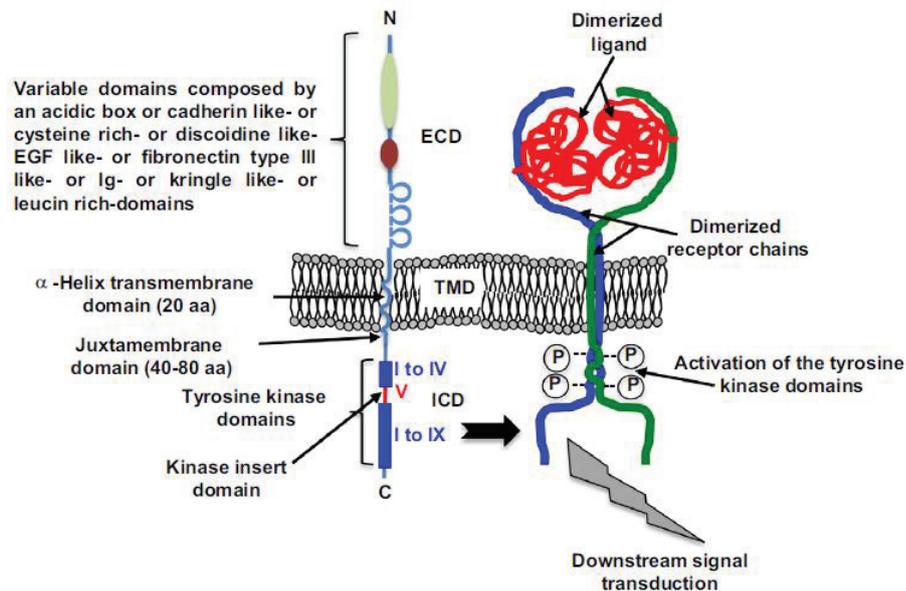
Receptor tyrosine kinases (RTKs) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. Protein kinases are key enzymes in the regulation of various cellular processes that catalyze the transfer of a phosphate group from ATP to a hydroxyl group of a serine or a threonine. Among the 90 identified genes encoding proteins with tyrosine kinase activity, 58 encode receptors are divided into 20 sub families (Robinson et al., 2000; Blume-Jensen et al., 2001) (Figure 2.1). Since the discovery of the first receptor tyrosine kinase (RTK) more than 30 years ago, many family members have emerged as key regulators of critical cellular processes, such as

proliferation and differentiation, cell survival and metabolism, cell migration, and cell-cycle control (Blume-Jensen et al., 2001; Ullrich and Schlessinger, 1990).

The RTK family members are characterized by a single trans-membrane domain and a glycosylated N-terminal extracellular domain with a high number of disulfide bonds. This extra-cellular domain is involved in ligand recognition and binding, followed by the dimerization process of the receptors (Figure 2.2). The composition of these domains (immune-globulin domains, rich in leucine, lysine and cysteine, fibronectin type III domain, etc.) depends on the classes of RTKs and then defines the specificity of the ligands (Figure 2.1). The RTKs are inserted into the cell membrane thanks to a  $\alpha$ -helix trans-membrane domain composed of 20 amino acids. The trans-membrane domain plays a key role in the formation and stabilization of the dimer of the receptor chains. In the lipid environment of the cell membrane, the  $\alpha$ -helices are non-covalently oligomerized (Arkin, 2002). This type of process makes it possible to pre-dimerize the RTKs in the cell membrane so that they are able to interact with the corresponding ligand (Moriki et al., 2001). The cytoplasmic domain harbors a specific region with tyrosine kinase activity that is involved in the catalysis of the ATP-dependent phosphorylation of receptor chains. Three regions compose the cytoplasmic domain: a juxta-membrane region composed of 40–80 amino acids, a tyrosine kinase domain and a carboxy-terminal region. The tyrosine kinase domain is composed of 12 subdomains organized into two lobes, connected by the kinase insert domain (sub-domain V) (Figure 2.2). The tyrosine kinase domain also includes an activation loop, whose orientation (and phosphorylation) determines the active or inactive state of the kinase domain.



**Figure 2.1** Receptor Tyrosine Kinase Families. Human receptor tyrosine kinases (RTKs) contain 20 subfamilies, shown here schematically with the family members listed beneath each receptor. Structural domains in the extracellular regions are marked, identified by structure determination or sequence analysis. The intracellular domains are shown as red rectangles (Lemmon and Schlessinger, 2010).

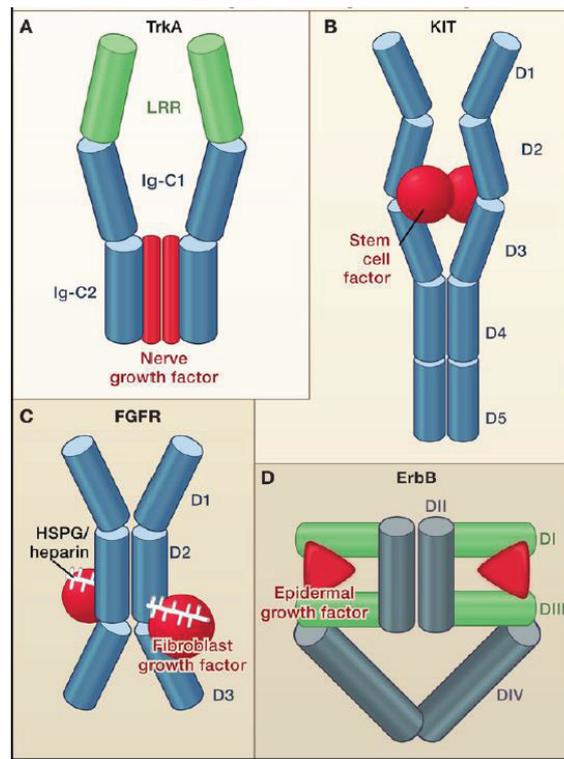


**Figure 2.2** General organization of the molecular domains that make up the RTKs. RTKs are characterized by the dimerization of two receptor chains with an N-terminal (N) extracellular domain (ECM), and a C-terminal (C) intracellular domain (ICD). The extracellular domain is implicated in the recognition of the dimeric ligands and the formation of the receptor chain dimerization process. The extracellular domain is associated with ligand recognition and is composed of various domains depending on the RTK class. The trans-membrane-domain is composed of a  $\alpha$ -helix chain, which contributes to the stabilization of the dimeric receptor chains. The binding of a dimeric ligand (in red) to the extracellular domains of the receptor chains strengthens the stabilization of the receptor chains, which are auto-phosphorylated through their tyrosine kinase domains and then transduced in specific downstream signaling pathways (Ségaly et al., 2015).

## 2.2 Ligands bind to RTKs and induce dimerization

In general, growth factor binding activates RTKs by inducing receptor dimerization (Ullrich and Schlessinger, 1990). Early studies of RTKs and cytokine receptors suggested a conceptually straightforward mechanism for ligand-induced dimerization: a bivalent ligand interacts simultaneously with two receptor molecules and effectively crosslinks them into a dimeric complex. This “ligand-mediated” mode of receptor dimerization was further supported by crystal structures of several fragments of the ligand-binding domains from RTKs bound to their relevant ligands. Recent structures of more complete extracellular regions of RTKs have provided important additional insight into the range of mechanisms used for ligand induced dimerization. Figure 2.3 illustrates two extreme mechanistic and two intermediate cases. At one extreme, receptor dimerization is entirely

“ligand mediated” and the two receptors make no direct contact (Figure 2.3A). At the other extreme, dimerization is instead entirely “receptor mediated” (Figure 2.3D), and the ligand makes no direct contribution to the dimer interface. Alternatively, dimerization can involve a combination of ligand-mediated and receptor-mediated components (Figure 2.3B and 2.3C). Dimerization of most RTKs is likely to resemble one of these four modes. However, additional paradigms are likely to emerge from more comprehensive studies of other RTK families.



**Figure 2.3** Four modes of receptor tyrosine kinase dimerization (Lemmon and Schlessinger, 2010).

### 2.3 Mechanism of RTKs activity

It is well known that the binding of a dimeric ligand to its receptor chains increases the proximity or/and stabilizes the receptor chains that will be then auto-phosphorylated through their kinase domains (a process called trans-phosphorylation). This non-covalent dimerization is associated with conformational changes that lead to the activation of the cytoplasmic kinase domains of the receptors. In most cases, one of the two receptor

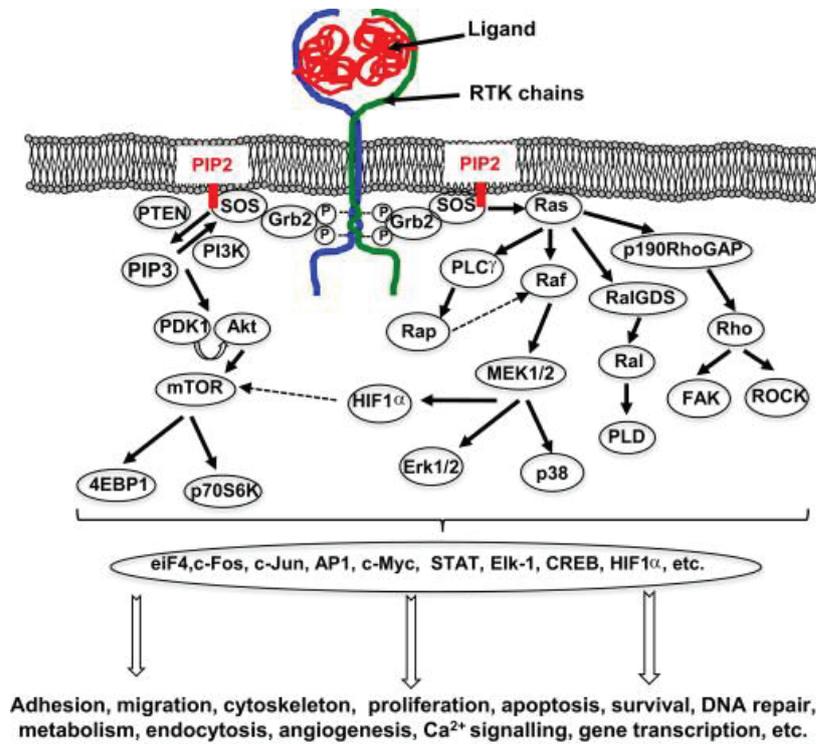
chains will trans-phosphorylate specific cytoplasmic tyrosine from other monomeric chain environment (Lemmon and Schlessinger, 2010).

In the absence of the ligand, the activation loop self-regulates activation of the receptor as its “closed” conformation inhibits catalytic activity (cis-inhibition). Dimerization of the RTK chains following ligand binding induces the rotation of the N- and C-lobes, as well as the major axis of the protein. The activation loop, which is masked by its tyrosine residues, the ATP binding site, moves to enable ATP binding and the autophosphorylation of tyrosine residues located on the opposite receptor chain. The trans phosphorylation of key tyrosine residues located in the activation loop stabilizes the “open” conformation and breaks the binding between these tyrosines and the binding sites of the protein substrates, making it possible to access the C lobe, then activating its kinase activity. In addition, other tyrosine residues are phosphorylated by protein kinases previously recruited on the phosphorylated tyrosines of the RTK environment (Hubbard, 2002).

Phosphorylation of the catalytic domain of the RTKs activates and increases the activity of the kinase domain, whereas the non-catalytic domains create various anchoring sites for cytoplasmic targets involved intracellular signal transduction. These tyrosines are mostly located on the juxta-membrane and C-terminal domains, and at the insert kinase domain residues, allowing the binding, activation and phosphorylation of numerous cytoplasmic proteins that will then release the signal towards various intracellular activation pathways. These proteins have SH2 or PTB domains that recognize tyrosine phosphorylated receptor chains, and have intrinsic enzymatic activity, such as Src or PLC $\gamma$ , or serve as adapter proteins for recruiting other enzymes, such as Grb2 linked to the MAPK activation pathway. The proteins recruited by their SH2 domains are named “adapter”, while those that bind directly to the receptor chains or to the Grb2 adaptive protein are called “anchoring proteins”. Adaptive and anchoring proteins can bind to similar phosphorylated tyrosine residues or to several tyrosine residues from the same receptor chains.

RTKs are considered as protein platforms, or the starting point for many cellular signaling pathways by recruiting enzymatic effectors (PLC $\gamma$ , PI3K, Src, etc.) either directly on to their intra-cytoplasmic domain, or indirectly through adapter proteins (Grb2, Shc, etc.), forming complexes capable of activating intracellular enzymes (Ras, etc.) (Figure 2.4). RTK downstream signaling pathways are mainly MAPK, PI3K, Src, and other signaling pathways involving PLC $\gamma$ , JAK / STAT, etc. While the early stages of signal

transduction following the activation of RTKs is based mainly on tyrosine phosphorylation, signal propagation associates various phosphorylation on serine/threonine residues in the majority of cellular processes, as well as other processes such as ubiquitination, glycosylation or acetylation (Choudhary and Mann, 2010).



**Figure 2.4** Main signaling pathways activated by the ligand-induced RTK auto-phosphorylation. The phosphorylation cascades initiated by the RTK phosphorylation lead to the activation of numerous transcription factors, which consequently control the regulation of many physiological processes (Ségallyn et al., 2015).

## 2.4 Downstream signaling pathways of RTKs

RTKs have numerous downstream signaling pathways, implicating in regulation of various proteins. Here, the several major pathways will be discussed.

The first downstream signaling pathway that follows RTKs activation is the MAPK (Mitogen-activated protein kinase) pathway, which plays a role in controlling cell proliferation, survival, differentiation, migration and promoting angiogenesis. The MAPK signaling cascade is divided into four major pathways used by RTKs and leading to ERK1/2 activation. After activation of the RTKs by their ligand, the adaptive protein Grb2

binds by its SH2 domains, the phosphorylated tyrosine residues of the receptor chains and the adaptive protein Son of Sevenless (SOS, a Guanine nucleotide exchange factors (GEF) protein exchanging the GDP for a GTP), by their SH3 domain, which is bound to the PIP2 membrane. This binding allows the activation of Ras, a small G protein, *via* SOS. In fact, Ras oscillates between its active and inactive state, thus acting as a “switch” for intracellular effector molecules. Once activated, Ras allows phosphorylated signal transduction through recruitment and phosphorylation of Raf kinases A, B or C (or MAP3K) (Cseh et al., 2014). Activated Raf phosphorylates MEK1 and MEK2 (or MAP2K1/2) on serine218/serine222 and serine222/serine226 residues of their activation loop, then activated MEK1/2 itself catalyzes the phosphorylation of Erk1 and Erk2 (or MAPK1/2) on their threonine202/185 and tyrosine204/187 residues. Phosphorylated Erk1/2 will be then translocated to the nucleus to activate transcription factors that will regulate the transcription of genes involved in the survival and growth of the cells; or activate cytosolic proteins, such as RSK1/2, which will either target cytoplasmic effectors or finally be translocated into the nucleus to act as a transcription factor (Roskoski, 2012). The targets of these transcription factors are transcriptional regulators such as STAT, Elk-1, CREB or H3 histone that activate transcription of early genes. Of these early genes, c-Fos, c-Jun or c-Myc stimulate the expression of other genes such as cyclin D1 or CDK6, which control progression in the G1 phase and G1/S transition.

The second signaling pathway that can be triggered by RTKs is the PI3K/Akt/mTOR pathway, which controls cell cycle progression, the cell survival/apoptosis balance. Its activation facilitates several processes, such as cell proliferation and migration, and glucose metabolism. PI3K is a “lipid” kinase that phosphorylates membrane lipids via its catalytic p110 subunit ( $\alpha$ ,  $\beta$  or  $\delta$ ) once recruited by its two SH2 domains from the p85 regulatory subunit on activated RTKs. PIP2 then forms phosphatidylinositol 3,4,5-triphosphate (PIP3) by transferring a phosphate group, and Akt (also known as PKB, for Protein Kinase B) and Phosphoinositide-dependent kinase-1 (PDK-1) then bind to the membrane, where the PDK-1 is activated by PIP3 phosphorylates Akt. Activated Akt becomes an activation crossroad for many proteins, allowing cells to survive by inhibiting, ubiquitinating and degrading pro-apoptotic proteins such as BAD and p53, and by inducing the expression of anti-apoptotic proteins such as Bcl-2. In addition, Akt also induces cell proliferation by activating various cyclins and by inhibiting several cell cycle repressors such as p21 or p27. Akt also allows the transcription of pro-angiogenic genes such as VEGF and HIF-1 $\alpha$ , which are involved in numerous oncological processes. In addition, Akt

inhibits the glucose metabolism by suppressing GSK3, and regulates the lipid metabolism through mTOR activation (Song et al., 2005).

The role of the Src pathway in signal transmission after RTK activation within the cell was demonstrated for the first time in fibroblasts stimulated with PDGF (Ralston et al., 1985). Src, Fyn and Yes belong to the Src family, activated by RTKs, and are associated with numerous other kinases such as Ras, PI3K, PLC $\gamma$  or FAKs. The members of the Src family therefore have redundant functions in the intracellular signaling pathways. Src family members are recruited on RTKs (EGFR, FGFR, IGFR, MCSF-R, HGFR, etc.) after their activation and transmit mitogen signals inducing DNA synthesis, cell survival, cytoskeleton rearrangements, cell adhesion and motility, as well as control receptor turnover (Bromann et al., 2004). Src family members can bind phosphorylated residues by their SH2 domains, resulting in kinase activity after conformational modifications. This activation is very complex and requires the recruitment of Ras and Ral GTPases. Several studies have shown that SFKs may regulate activation of RTKs directly by phosphorylating tyrosine residues such as tyrosine845, tyrosine1101 and EGFR (Biscardi et al., 1999). c-Src can be recruited within membrane complexes formed by integrins, and then phosphorylate these RTKs (Moro L et al., 2002). Furthermore, the Shp2 protein tyrosine phosphatase also plays a key role in this activation by blocking the activities of negative regulators (Csk for instance) (Goi et al., 2000).

PLC $\gamma$  and JAK/STAT are additional signaling pathways associated with RTK activation. Various RTKs can bind through their phosphorylated tyrosine residue, the SH2 domains of STAT transcription factors, as demonstrated for MET and STAT3. The activation of these transcription factors results in their dimerization and translocation into the nucleus to activate specific target genes (Trusolino et al., 2010).

## **2.5 Receptor tyrosine kinase and cancer**

In contrast to the regular proliferation and differentiation of development, cancer represents an accumulation of genetic and epigenetic changes resulting in a disregard for the constraints of differentiation, proliferation, programmed cell death and localization. By the time cancers reach an advanced state, genomic instability often results in hundreds of mutations, which can be categorized as either 'driver' mutations, those conferring a

selective growth advantage to cells and are instrumental in cancer initiation or progression, or ‘passenger’ mutations, which do not contribute to oncogenesis.

Protein kinases, including RTKs, are one of the most frequently mutated gene families implicated in cancer, which has prompted numerous studies on their role in cancer pathogenesis (Blume-Jensen and Hunter, 2001; Lahiry et al., 2010; Torkamani et al., 2009). There are four main mechanisms of RTKs dysregulation in human cancers: genomic rearrangements, autocrine activation, overexpression and gain- or loss-of-function mutations (Blume-Jensen and Hunter ,2001; Lemmon and Schlessinger,2010). Unchecked RTK signaling can disrupt the balance between cell growth, cell-cycle progression and apoptosis. At some circumstance, the deregulated RTKs may sensitize cells to oncogenic transformation or trigger RTK-induced oncogenesis (Lemmon and Schlessinger,2010; Pon and Marra, 2015). Table 2.1 shows the implicated RTKs in different types of cancers.

Oncogenic RTK (Examples)	Chromosome Location	Cancer (Examples)	Approved Selective TKI for Treatment
ALK	2p23	NSCLC, colorectal cancer, breast cancer	-
AXL	19q13.1	Lung, colon, breast, AML, CML	-
CCK4 (PTK7)	6p21.1	small cell lung cancer, breast cancer, gastric and colon cancer, AML	-
DDR1	6p21.33	NSCLC, breast cancer, AML, ovarian cancer	-
DDR2	1q23.3	NSCLC, lung cancers, CML, breast cancer	-
EGFR1 (ERBB1/HER1)	7p11.2	Breast cancer, hepatocellular carcinoma	+
EGFR2 (ERBB2/HER2)	17q12	Breast cancer, gastric adenocarcinomas	+
EGFR3 (ERBB3/HER3)	12q13.2	Breast cancer, ovarian cancer, Squamous cell lung cancer	+
EGFR4 (ERBB4/HER4)	2q34	Breast cancer, melanoma	+
EPHA1	7q35	NSCLC, prostate cancer	-
EPHA2	1p36.13	Hepatocellular carcinoma, colorectal cancer, breast cancer	-
EPHA3	3p11.1	Glioblastoma, lung cancer, melanoma, ALL	-
EPHA4	2q36.1	NSCLC, gastric cancer	-
EPHA5	4q13.1	Breast cancer, hepatocellular carcinoma, ALL	-
EPHB1	Xq13.1	NSCLC, cervical cancer, ovarian Cancer	-
EPHB2	13q33.3	Cervical cancer, breast cancer	-
EPHB3	3q27.1	NSCLC, breast cancer, colorectal cancer	-
EPHB4	7q22.1	Breast cancer, melanoma, glioma	-
FGFR1	8p12	Squamous cell lung cancer, breast cancer	-
FGFR2	10q26	Squamous cell lung cancer, breast cancer, thyroid cancer	-

Oncogenic RTK (Examples)	Chromosome Location	Cancer (Examples)	Approved Selective TKI for Treatment
FGFR3	4p16.3	Bladder cancer, squamous cell carcinoma	-
FLT3	13q12.2	AML, acute promyelocytic leukemia	-
IGF1R	15q26.3	CLL, breast cancer, pancreatic cancer	-
IGF2R	6q25.3	breast cancer, prostate cancer, colorectal carcinoma	-
INSR	19p13.2	Colorectal cancer, prostate cancer	-
INSRR	1q23.1	Neuroblastoma	-
KIT	4q12	AML, melanoma, ovarian carcinoma	-
LTK	15q15.1	Gastric cancer, lymphomas and leukemias	-
MER	2q13	Glioblastoma, hepatocellular carcinoma	-
MET	7q31.2	Hepatocellular carcinoma, CLL, breast cancer	-
MUSK	9q31.3	Ovarian cancer	-
NTRK1 (TrkA)	1q21-22	Colorectal cancer, breast cancer	-
NTRK2 (TrkB)	9q22.1	Neuroblastoma, astrocytoma	-
NTRK3 (TrkC)	15q25	Neuroblastoma, breast cancer	-
PDGFRA	4q12	Lung adenocarcinoma, gastrointestinal stromal tumors	-
PDGFRB	5q32	gastrointestinal stromal tumors, glioblastoma	-
RET	10q11.2	NSCLC, medullary thyroid carcinoma	-
RON (MST1R)	3p21.31	Pancreatic cancer, breast cancer, NSCLC	-
ROR1	1p31.3	CLL, ALL, AML, MCL, HCL, melanoma	-
ROR2	9q22.31	Melanoma, hepatocellular carcinoma, colon cancer	-
ROS1	6q22	NSCLC, ovarian cancer	-
RYK	3q22.2	CML, ovarian cancer	-
TIE	1p34.2	Glioblastoma, breast tumor	-
TEK	9p21.2	Bladder cancer, glioblastoma, AML	-
TYRO3	15q15.1	Colon cancer, melanoma, thyroid cancer, breast cancer	-
VEGFR1 (FLT1)	13q12.3	Ovarian cancer, NSCLC, colorectal carcinoma	+
VEGFR2 (Kdependence receptor)	4q12	Renal cell carcinoma, breast cancer	+
VEGFR3 (FLT4)	5q35.3	Thyroid carcinoma, breast cancer	+

**Table 2.1** Oncogenic receptor tyrosine kinases in cancer (Hojjat-Farsangi, 2014).

ALK: anaplastic lymphoma receptor tyrosine kinase, NSCLC: non-small cells lung carcinoma, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, DDR: Discoidin domain receptor, EGFR: epidermal growth factor receptor, EPHA: ephrin type-A receptor, ALL: acute lymphoid leukemia, EPHB: ephrin type-B receptor, FGFR: fibroblast growth factor receptor, FLT3: Fms-like tyrosine kinase 3, IGF1R: insulin growth factor receptor, CLL: chronic lymphocytic leukemia, INSR: insulin receptor, LTK: leukocyte tyrosine kinase, NTRK: neurotrophic tyrosine kinase, PDGFR: platelet-derived growth factor receptor, ROR: receptor tyrosine kinase-like orphan receptor, VEGFR: vascular endothelial growth factor receptor.

## 2.6 Targeting RTKs by tyrosine kinase inhibitors

Tyrosine kinase inhibitors (TKIs), as well as other small inhibitors, are low molecular weight organic compounds. The molecular weight of those small inhibitors should better be more than 500 Daltons, based on the observation that clinical attrition rates are significantly reduced when the molecular weight falls below 500 Daltons (Owens, 2003; Lipinski, 2004). The upper molecular weight is approximately 900 Daltons (Veber et al. 2002).

Proper TKIs are usually selected by high-throughput screening (HTS) methods that detect the most proper TKI candidates among a large library of compounds. An optimal TKI should have particular characteristics for further development. Absorption, distribution, metabolism, excretion, and toxicity (ADMET) of a drug candidate are the most important elements that should optimize for in vivo use (Prueksaritanont and Tang, 2012). TKIs prevent and block vital pathways through targeting signaling molecules which are necessary for cell survival. TKIs can translocate through the plasma membrane and interacts with the cytoplasmic domain of RTKs. After this interaction, TKIs inhibit the catalytic activity of the TK domain by interfering with the binding of ATP or its substrates (Johnson, 2009).

TKIs are classified into three main groups. Most of the current TKIs are ATP-competitive inhibitors and are classified as type I inhibitors. Due to the highly conservative ATP-binding sites in TK domains and a high rate of competition with intracellular ATP, several difficulties obstruct the development of specific/selective TKIs of type I. Besides, TKIs might target other kinases, thereby suggesting that the anti-tumor effects may be due to the effects on other signaling molecules. Types II and III are non-ATP competitors and act through induction of structural changes in the RTKs. The conformational shifts modify the TK domain in a way that the TK domain loses its kinase activity (Garuti, 2010). Moreover, these inhibitors can bind to residues within the TK domain and prevent tyrosine phosphorylation (Tables 2.2 and 2.3).

Name	Trade/Code Name	Mol. Mass (g/mol)	Selective Target	IC <sub>50</sub> (nM/L) *	FDA Approved	Cancer (Examples)
Afatinib	Gilotrif	485.94	HER2, EGFR	0.5, 14	+	NSCLC, squamous cell carcinoma of the head and neck, breast cancer
Canertinib	CI-1033	485.94	EGFR, HER2, 4	0.8, 19, 7	-	Head and neck, breast, and NSCLC, ovarian cancer
Cediranib	Recentin	450.5	VEGFRs	<1	-	NSCLC, kidney and colorectal cancer
CP-673451	-	417.5	PDGFRs	<1	-	NSCLC, colon carcinomas, glioblastoma
Crizotinib	Xalkori	450.34	MET	11	+	NSCLC, anaplastic large cell lymphoma, neuroblastoma
Crenolanib	CP-868-596	443.54	MET, ALK, FLT3, PDGFR $\alpha,\beta$	11, 24, 0.74, 1, 0.4	-	AML, gastrointestinal stromal tumor, glioma
Dacomitinib	PF-00299804	469.94	EGFR	6	-	NSCLC, gastric, head and neck cancer, glioma
Erlotinib	Tarceva	393.43	EGFR	2	+	NSCLC, pancreatic cancer
EMD1214063	-	492.57	MET	3	-	NSCLC
EMD1204831	-	-	MET	9	-	NSCLC
Gefitinib	Iressa	446.9	EGFR	<57	+	NSCLC, AML
Icotinib	Conmana	391.15	EGFR	5	+	NSCLC
KW-2449	-	332.4	FLT3	6.6	-	AML
Lapatinib	Tykerb	581.06	HER-2, EGFR	9.2, 10.8	+	Breast cancer
Lenvatinib	E7080	426.85	VEGFR2, 3	<4	+	Approved for thyroid cancer in Japan
LY2801653	-	552.53	Met, RON	<2	-	NSCLC
Neratinib	HKI-272	557.04	EGFR, HER2	92, 59	-	NSCLC, breast cancer
PD-173074	-	523.67	FGFRs	<25	-	NSCLC, gastric carcinoma, breast cancer
Quizartinib	AC220	560.67	FLT3	<4.2	-	AML
R428	BGB-324	506.64	AXL	14	-	AML, NSCLC, breast cancer
Tandutinib	MLN518/CT53518	562.7	FLT3	<100	-	RCC, CML
Tivantinib	Arqule/ARQ-197	369.42	MET	4	-	RCC, breast cancer
Tivozanib	AV-951	454.86	VEGFR1, 2, 3	0.21, 0.16, 0.24	-	RCC, breast cancer
Vatalanib	PTK787/ PTK/ZK	346.81	VEGFR2	37	-	NSCLC, DLBCL, colorectal adenocarcinoma

**Table 2.2** Current specific/selective tyrosine kinase inhibitors (TKIs) targeting receptor tyrosine kinases (RTKs) (Hojjat-Farsangi, 2014).

\* Half maximal inhibitory concentration (IC<sub>50</sub>) values are the measure of the effectiveness of TKIs in inhibiting the RTKs in biochemical assays, HER: human epidermal receptor, EGFR: epidermal growth factor receptor, NSCLC: non-small cells lung carcinoma, VEGFR: vascular endothelial growth factor receptor, PDGFR: platelet-derived growth factor receptor, ALK: anaplastic lymphoma receptor tyrosine kinase, FLT3: Fms-like tyrosine kinase 3, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, RCC: renal cell carcinoma, DLBCL; Diffused large B-cell lymphoma.

Name	Trade/Code Name	Mol. Mass (g/mol)	Target Molecules (Examples)	IC <sub>50</sub> (nM/L) *	FDA Approved	Cancer (Examples)
Amuvatinib	MP470	447.51	ALK, MER, KIT, RET, PDGFRs, FLT3, RAD 51	<100	-	NSCLC
Axitinib	Inlyta	386.5	VEGFRs, PDGFRs, KIT	<1.7	+	RCC
Cabozantinib (XL184)	Cometriq	501.51	VEGF, RET, MET, NTRKB, TIE2, AXL	<15	+	Medullary thyroid cancer, progressive metastatic medullary thyroid cancer
Dasatinib	Sprycel	488.01	BCR-ABL, SRC, KIT, PDGFRs, EPH, CSK	<10	+	CML, ALL
Foretinib	-	632.65	VEGFR2, MET	0.9, 0.4	-	NSCLC, breast, gastric, papillary renal cancer
Golvatinib	E7050	633.69	VEGFR2, MET	16, 14	-	Gastric cancer, HCC, glioblastoma, melanoma
Imatinib	Gleevec	589.7	ABL, KIT, PDGFRs	0.6, 0.1, 0.1	+	Gastrointestinal stromal tumor, leukemias
MGCD-265	-	517.6	MET, VEGFRs, TIE2, RON	<7	-	NSCLC
Nilotinib	Tasigna	529.5	BCR-ABL, KIT, LCK, EPHA3, 8, DDR1, 2	<30	+	CML
Pazopanib	Votrient	437.51	PDGFRs, VEGFRs	<150	+	Advanced renal cell carcinoma, advanced soft tissue sarcoma
Ponatinib	Iclusig	532.56	BCR-ABL, PDGFR $\alpha$ , SRC, KIT, FGFR, VEGFRs	<6	+	CML, philadelphia chromosome positive ALL
Regorafenib	Stivarga	482.82	TIE2, PDGFRs, RET, KIT, B-RAF	<25	+	Metastatic colon cancer
Sorafenib	Nexavar	464.8	VEGFRs, PDGFRs, B-RAF, MEK, ERK	<100	+	Advanced renal cell carcinoma, hepatocellular carcinoma
Sunitinib	Sutent	532.56	VEGFR2, PDGFR $\beta$ , KIT, RET, CSF1R, FLT3	<100	+	Renal cell carcinoma, gastrointestinal stromal tumor
Vandetanib	Caprelsa	475.35	EGFR, VEGFRs, RET, Tie-2, FGFR1	<500	+	Metastatic medullary thyroid cancer

**Table 2.3** Multi-targeted tyrosine kinase inhibitors (TKIs) targeting RTKs and intracellular kinases (Hojjat-Farsangi, 2014).

\* Half maximal inhibitory concentration (IC<sub>50</sub>) values are the measure of the effectiveness of TKIs in inhibiting the RTKs in biochemical assays, ALK: anaplastic lymphoma receptor tyrosine kinase, FLT3: Fms-like tyrosine kinase 3, PDGFR: platelet derived growth factor receptor, EGFR: epidermal growth factor receptor, NSCLC: non-small cells lung carcinoma, VEGFR: vascular endothelial growth factor receptor, VEGF: vascular endothelial growth factor, NTRK: neurotrophic tyrosine kinase, EPHA: ephrin type-A receptor, Ddependence receptor: Discoidin domain receptor, CML: chronic myeloid leukemia.

### **3. Receptor tyrosine kinases (RTKs) and dependence receptors (DRs)**

#### **3.1 From RTKs to DRs**

As mentioned in the introduction text of dependence receptors, more than 20 receptors have been identified as dependence receptors, sharing the ability to induce cell death in the absence of their respective ligand. Of interest, those receptors include some receptors tyrosine kinase: TrkA, TrkC, MET, RET and EphA4, which have been shown to act as dependence receptor (Nikoletopoulou et al., 2010; Tauszig-Delamasure et al., 2007; Tulasne et al., 2004; Bordeaux et al., 2000; Furne et al., 2009). It is known that, just like other RTKs, those receptors (TrkA, TrkC, MET, RET and EphA4) depend on their ligand to induce positive signaling (such as cell survival and growth). Thus, it is both surprising and interesting that they are also able to induce apoptotic signaling when their ligands are absent. In the following text, we will have a look at the dependence receptor features of those three well studied members: MET, RET and TrkC.

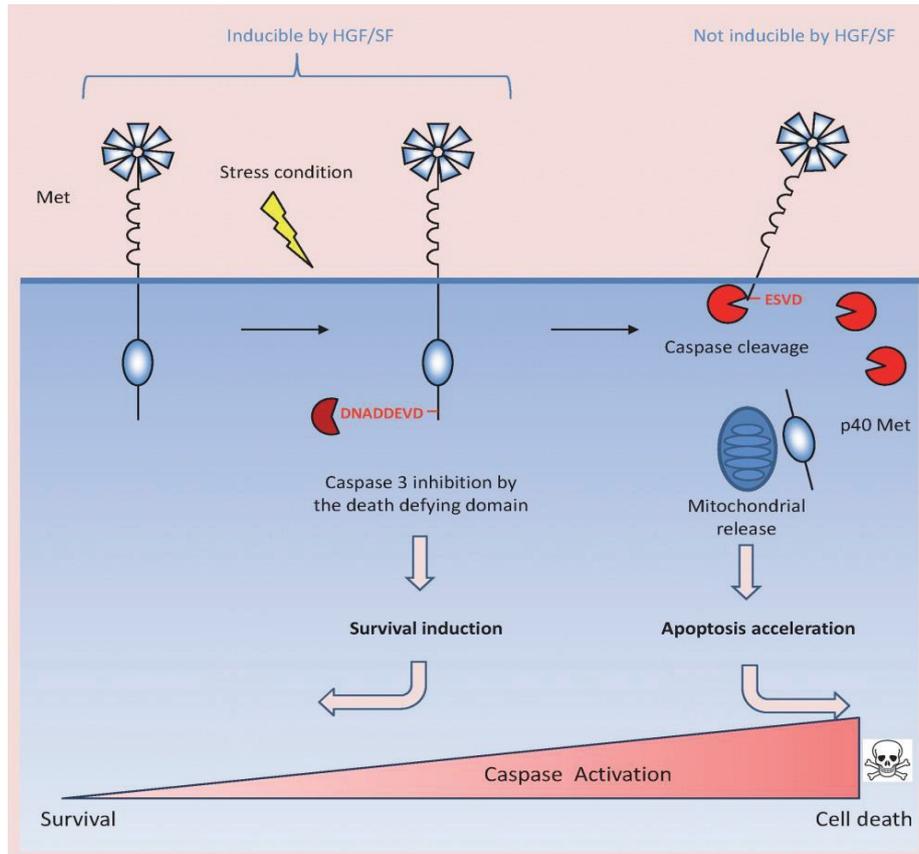
#### **3.2 MET leads to apoptosis by releasing p40 cleavage fragment**

MET, also called tyrosine-protein kinase Met or hepatocyte growth factor receptor (HGFR), is a receptor tyrosine kinase expressed predominantly by epithelial cells and activated by its stromal ligand, hepatocyte growth factor/scatter factor (HGF/SF). MET activation stimulates a biological program called invasive growth, involving in survival, proliferation, invasion, and morphogenesis of epithelial cells (Trusolino et al., 2010). Ligand stimulated MET acts further as an angiogenic and neurotrophic factor (Bussolino et al, 1992; Maina et al., 1999). HGF/SF and MET are essential to several steps of embryogenesis, experiments on transgenic mice having shown that they are necessary for formation of the placenta, liver, limb muscle, neurons, and lung airspace (Uehara et al., 1995; Schmidt et al., 1995; Bladt et al., 1995; Maina et al., 1997). Similar to many other RTKs, aberrant MET and HGF/SF signaling contributes to promoting tumorigenesis and metastasis.

However, recent findings demonstrated that MET can regulate survival/apoptosis balance through an unexpected mechanism. On one hand, it has been shown that human MET receptor displayed a double consensual caspase site at its C-terminal end, which is able to inhibit caspase-3 activity. This direct caspase-3 inhibition contributes to survival of

hepatocytes in cell culture and animal models, reinforcing the anti-apoptotic function of the receptor. On the other hand, it has also been demonstrated that, during apoptosis, MET is cleaved by caspases at another site located in the juxtamembrane region (Tulasne et al., 2004). These cleavages remove the C-terminal tail of MET and separate the extracellular ligand-binding domain from the intracellular kinase domain. The generated 40-kDa intracellular fragment can increase cell death by promoting mitochondrial permeabilization (Foveau et al., 2007; Lefebvre et al., 2013). Removal of the C-terminal tail of MET is required for the efficient pro-apoptotic action of the fragment.

The proposed model states that in the early steps of apoptosis, the MET C-terminal caspase site is able to protect cells from apoptosis by inhibiting caspase-3 activity. In this condition, the MET receptor is still inducible by its ligand and potentially able to promote survival (Figure 3.1). In the later steps of apoptosis, when caspase activation reaches a higher level, MET is then cleaved at the juxtamembrane site leading to generation of a 40-kDa proapoptotic fragment able to promote mitochondrial permeabilization. At this stage, MET is not any longer inducible by its ligand. Therefore, MET could regulate survival/apoptosis balance through opposite functions according to the level of caspase activity (Figure 3.1). This pro-apoptotic function that dependent on the ligand of MET makes it a member of the dependence receptor family.



**Figure 3.1** Model of the survival/apoptosis balance regulation by the MET receptor. In the early steps of apoptosis, the C-terminal caspase site of Met DNADDEVD can inhibit caspase-3 activity, contributing to survival of epithelial cells. At this stage, ligand-dependent survival is still effective. At later stages of apoptosis, stronger caspases activation can lead to juxtamembrane cleavage of MET at the ESVD site, generating a 40-kDa fragment (p40 Met) involved in mitochondrial permeabilization and acceleration of apoptosis. At this later stage, MET is not inducible any longer by HGF/SF (Furlan and Tulasne, 2014).

### 3.3 RET acts as a dependence receptor through RET/Pit-1/p53 pathway

RET (rearrangement during transfection) has been classically considered as a proto-oncogene. The RET proto-oncogene encodes a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family (Knowles et al., 2006). RET loss of function mutations are associated with the development of Hirschsprung's disease, while gain of function mutations are associated with the development of various types of human cancer, including medullary thyroid carcinoma, multiple endocrine neoplasias type 2A and 2B, pheochromocytoma and parathyroid hyperplasia (Qi et al., 2011).

However, new pathway of RET has been discovered. Indeed, in the absence of GDNF, RET is cleaved by caspase-3 like caspase in position D707 and D1017, forming a complex with PKC $\delta$  and caspase-3 in the cytoplasmic site of the membrane. Once complexed, the three become processed and caspase-3 and PKC $\delta$  become active. Except for the direct activation of caspase-3 that can induce apoptosis, the activation of PKC $\delta$  also induces the binding of cEBP $\alpha$ /CREB to an element of the Pit-1 promoter conserved in mouse, rat and humans with a huge induction of Pit-1 (Bordeaux et al., 2000; Canibano et al., 2007; Garcia-Lavandeira et al., 2010). Pit-1 over-expression leads to p53 accumulation and apoptosis. Further research shows that activation of the RET/Pit-1/p53 pathway is able to block tumor growth in vivo (Diaz-Rodriguez et al., 2012).

These findings indicate that RET, like other dependence receptors, could be a two-sided tumor regulator: a tumor suppressor because of its dependence receptor activity and an oncogene because of its tyrosine kinase activity. These results also raise the possibility of RET-based therapy as a treatment for conventional Pit-1-expressing chemotherapy-resistant adenomas.

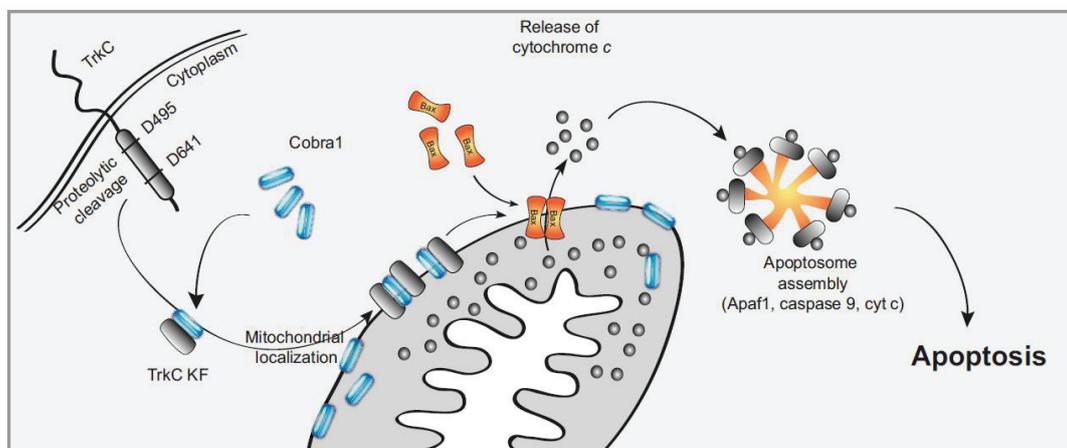
### **3.4 TrkC triggers mitochondrial apoptosis pathway through its “killer fragment”**

Tropomyosin receptor kinase C (TrkC), also known as NT-3 growth factor receptor, neurotrophic tyrosine kinase receptor type 3, or TrkC tyrosine kinase, is a protein that is encoded by the NTRK3 gene in humans. TrkC is the high affinity catalytic receptor for the neurotrophin-3 (NT-3). Neurotrophins include NGF, BDNF, NT-3, and NT-4 (Huang and Reichardt, 2001). These proteins have been shown to be crucial for the development of the nervous system, especially by controlling the massive developmental loss of neurons that are produced in excess and fail to adequately connect their targets. The current neurotrophic model holds that the main neurotrophin receptors, TrkA, TrkB, and TrkC, generate survival signals via the PI3K/Akt and Ras/MEK/MAPK pathways upon neurotrophin binding (Kaplan and Miller, 2000). This binding is thought to inhibit the naturally occurring apoptotic death of neurons.

Increasing evidence indicates that TrkC has some other roles except for RTK. It has been proven that in the absent of its ligand NT-3, TrkC is cleaved by caspases at two sites located at Asp-495 and Asp-641, generating a pro-apoptotic “killer fragment” (TrkC-KF) (Tauszig-Delamasure et al., 2007). TrkC-KF then interacts with Cobra1 (a putative

cofactor of BRCA1), which shuttles TrkC-KF to the mitochondria, where it promotes Bax activation, cytochrome c release, and apoptosome-dependent apoptosis (Ichim et al., 2013) (Figure 3.2). Recent study also shows that TrkC-KF is translocated in the nucleus via importins and interacts there with transcriptional factor Hey1. Hey1 and TrkC-KF interact and jointly bind to MDM2 promoter E-box, in which TrkC-KF favors Hey1 repressor function on MDM2 transcription. MDM2 transcriptional inhibition promotes p53 stabilization and thus apoptosis (Ménard et al., 2018).

In a large fraction of human colorectal cancers, TrkC expression is down-regulated, mainly through promoter methylation, which is a selective advantage for colorectal cell lines to limit tumor cell death. Besides, reestablished TrkC expression in colorectal cancer cell lines is associated with tumor cell death and inhibition of in vitro characteristics of cell transformation, as well as in vivo tumor growth. Further, a mutation of TrkC detected in a sporadic cancer is found to be a loss-of proapoptotic function mutation. Thus, TrkC is a putative tumor suppressor in colorectal cancer, corresponding to its dependence receptor features, rather than receptor tyrosine kinase features (Genevois et al., 2013).



**Figure 3.2** A model of Cobra1's involvement in TrkC-induced apoptosis via the mitochondrial intrinsic pathway. The unliganded TrkC is cleaved by caspases, releasing TrkC-KF, which localizes at the mitochondria. This shuttling seems to require Cobra1. Furthermore, TrkC-KF is able to release cytochrome c and, thus, initiates the classical cascade of the intrinsic apoptosis (Ichim et al., 2013).

## 4. Receptor c-Kit and its ligand SCF

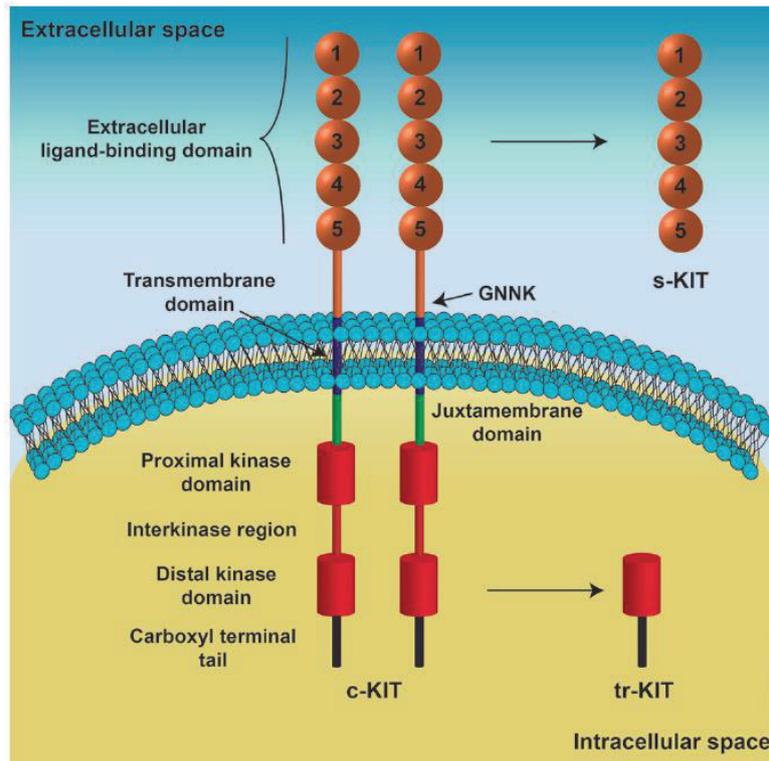
### 4.1 Overview of c-Kit

c-Kit is a tyrosine kinase receptor belonging to the type III receptor tyrosine kinase family, which also includes the platelet-derived growth factor receptor (PDGFR) and the macrophage colony stimulating factor receptor (Liu et al., 2007). Its specific ligand, stem cell factor (SCF), binds to the extracellular domain of c-Kit, inducing receptor activation and signal transduction.

c-Kit, also known as CD117, SCF receptor, or KIT receptor, was first described in 1986 as the transforming gene of the Hardy-Zuckerman 4 feline sarcoma virus and identified as the proto-oncogene v-Kit (Yarden et al., 1987), while the human ortholog is located on chromosome 4q1-q12. The structure of c-Kit displays three main functional domains (just as many other RTKs) (Figure 6.1): an extracellular ligand binding domain, a transmembrane region, and an intracellular kinase domain (Qiu et al., 1988; Blechman et al., 1995). The extracellular region, comprised of five immunoglobulin-like domains, recognizes the c-Kit ligand and also participates in receptor dimerization (Blechman et al., 1995; Paulhe et al., 2009). The tetrapeptide Gly-Asn-Asn-Lys (GNNK) in the extracellular juxtamembrane domain of c-Kit (Figure 6.1) helps regulate receptor activation and downstream signaling (Phung et al., 2013). A short chain of hydrophobic amino acids constitutes the transmembrane region that anchors c-Kit at the plasma membrane. The cytoplasmic region of c-Kit, which contains proximal and distal kinase domains separated by an interkinase domain (Figure 4.1), is responsible for transduction of SCF/c-Kit signaling (Mol et al., 2003; Roskoski, 2005).

c-Kit variants have been identified both in rodents and humans. Some isoforms arise through the use of alternative 5'-donor or 3'-acceptor splice sites in the c-Kit transcript, thereby producing proteins that differ by the presence or absence of the GNNK peptide (Hayashi et al., 1991) or a serine residue at position 715 in the interkinase domain (Reith et al., 1991; Caruana et al., 1999). The usage of alternative intronic promoter produces a 30-50 kDa truncated c-Kit protein (tr-KIT) that lacks the extracellular domain and the transmembrane region, remaining exclusively in the cytoplasm (Figure 6.1) (Rossi et al., 1992; Albanesi et al., 1996; Muciaccia et al., 2010). tr-KIT also lacks part of the kinase domain, so it does not have kinase activity (Rossi et al., 1992), although it seems to retain the ability to induce signaling through adapter proteins (Sette et al., 1998). Finally,

proteolytic cleavage of c-Kit generates a soluble isoform composed of the extracellular domain free of a transmembrane tether (Broudy et al., 1994; Turner et al., 1995). Soluble c-Kit binds SCF with the same affinity as the membrane anchored c-Kit, and so may help control the bioavailability of SCF (Wypych et al., 1995; Dahlen et al., 2001).



**Figure 4.1** Schematic representation of c-KIT. The five immunoglobulin-like domains of the extracellular region are involved in ligand-binding and receptor dimerization. The transmembrane domain anchors c-KIT in the cytoplasmic membrane. The intracellular region, responsible for signal transduction, contains proximal and distal kinase domains separated by an interkinase region and a carboxyl terminal tail. Some alternatively spliced forms of c-KIT are characterized by the presence of the tetrapeptide Gly-Asn-Asn-Lys (GNNK) in the extracellular juxtamembrane domain. The receptor can be cleaved and released from cell membrane, giving rise to a soluble c-KIT (s-KIT) consisting of only the extracellular domain. A truncated form of c-KIT (tr-KIT), derived from alternative promoter usage, lacks the extracellular and transmembrane domains but retains part of the kinase domain (Cardoso et al., 2014).

c-Kit is a protein consisting of 976 amino acids, making the core protein size 110 kDa. Heterogeneous N-linked glycosylation results in mature proteins appear between 145 and 160 kDa on protein gel (Qiu et al., 1988; Yarden et al., 1987). There are up to nine N-glycosylation sites, most of which are concentrated in extracellular domain close to the

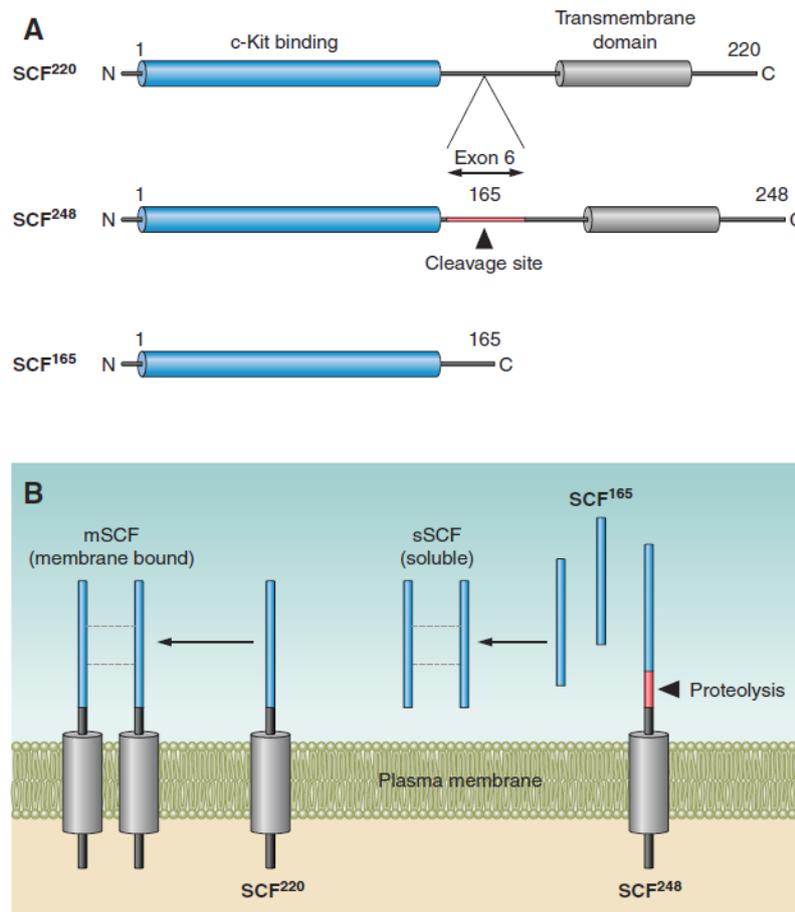
plasma membrane. Additional known post-translational modification of c-Kit include phosphorylation on both tyrosine and serine residues, both constitutive as well as ligand-induced serine phosphorylation (Blume-Jensen et al., 1994), that in some cases have been shown to function in fine tuning of receptor responses. Finally, ligand-induced ubiquitination of c-Kit is known to regulate both internalization and degradation of c-Kit (Masson et al., 2006; Sun, 2007; Zeng et al., 2005).

## **4.2 Overview of Stem Cell Factor (SCF)**

The c-Kit ligand is known by different names such as mast cell growth factor (Copeland et al., 1990; Anderson et al., 1990), kit ligand (Huang et al., 1990), steel factor and stem cell factor (SCF) (Zsebo et al., 1990). SCF is a non-covalent homodimer composed of two slightly wedged protomers where each protomer exhibits an anti-parallel four helix bundle fold (characteristic cytokine topology). Dimerization is mediated by polar and non-polar interactions between the two protomers with a large buried surface area (Zhang et al., 2000). Each SCF monomer contains two intra-chain disulphide bridges, Cys4–Cys89 and Cys43–Cys138 (Langley et al., 1992) (Figure 6.2B).

The SCF is encoded by the Steel (Sl) locus on chromosome 12 in the human and on mouse chromosome 10 (Anderson et al., 1991). It is encoded by 9 exons in human, mouse and rat (Martin et al., 1990) and present as both membrane bound (mSCF) and soluble (sSCF) forms (Anderson et al., 1990). The first SCF isoform is a 45 kDa /248 amino acid (aa) glycoprotein (SCF248) which is expressed at the cell membrane and cleaved by proteases to generate 31 kDa/ 165 aa soluble protein (sSCF or SCF165). The cleavage site: Val–Ala–Ala–Ser, aa 163–166 is encoded by exon 6 (Majumdar et al., 1994). The second SCF isoform is generated by alternate splicing around exon 6 (Williams et al., 1992). This isoform is 32 kDa/ 220 aa glycoprotein lacking exon 6 and remains membrane bound (mSCF or SCF220). There are also reports indicating that this membrane bound SCF may also be cleaved (Huang et al., 1992). The secondary cleavage site used to generate the soluble form is located in exon 7 in mouse and is used in the absence of primary cleavage site. Murine cell lines with mutated primary and secondary cleavage sites produced only membrane bound SCF showing absence of other major cleavage sites (Majumdar et al., 1994). Human SCF is translated with a 25 aa leader sequence followed by a 185 aa extracellular sequence, a 27 aa transmembrane region and a 30 aa intracellular region. Though activation of c-Kit by SCF is highly species specific, it is of interest that murine SCF shows 83% homology to human SCF (Williams et al., 1992). The

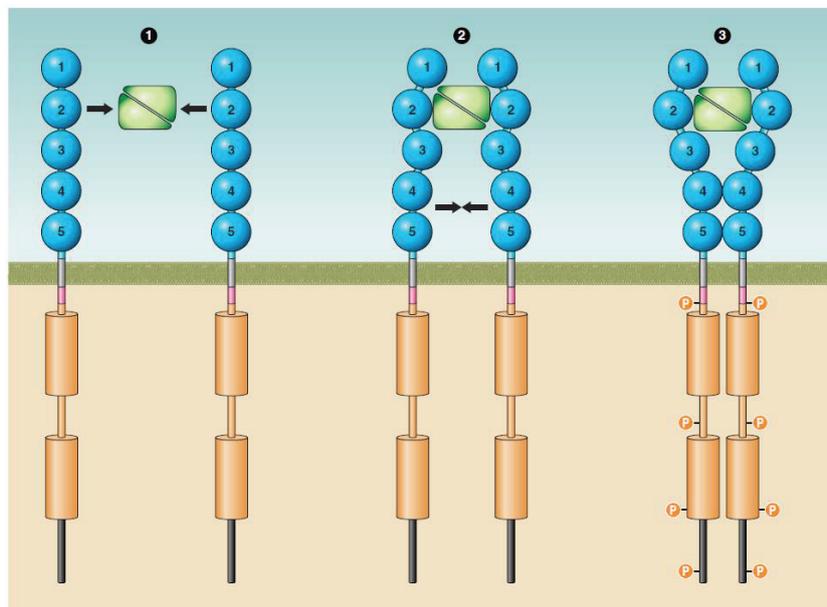
N terminal 141 residues of SCF have been identified as a functional core, SCF1–141, which includes the dimer interface and portions that bind to c-Kit (Langley et al., 1994). The SCF binding to kit induces a rapid and complete receptor dimerization leading to activation (by autophosphorylation) of the catalytic tyrosine kinase for signal transduction (Lev et al., 1992) (Figure 4.2 A,B).



**Figure 4.2** Schematic representation of stem cell factor (SCF) splice forms and protein processing. A: SCF protein is produced as two transmembrane forms due to alternative splicing of exon 6, SCF220, and SCF248. In SCF248, exon 6 is kept and encodes a proteolytic cleavage site, generating the soluble SCF165. B: SCF220, lacking the cleavage site, forms membrane-bound SCF dimers (mSCF), and SCF248 is processed to SCF165 that forms soluble SCF (sSCF). Dashed lines indicate that the SCF dimers are held together by noncovalent interactions (Lennartsson and Rönstrand, 2012).

### 4.3 c-Kit activation by SCF binding

The activation mechanism of c-Kit receptor was studied by Yuzawa et al., 2007. The c-Kit extra-cellular domain composed of five Ig (immunoglobulin)-like domains (indicated as D1-5). This first 3 domains (D1-3) are the domains that interact directly with ligand SCF. Concerning D4, the strong electrostatic environment of D4 can induce repulsion between D4-D4 and maintain the inactive monomer. As both D4-D4 and D5-D5 binding affinity is respectively lower in the absence of SCF, c-Kit extracellular domains are not easy to form dimers. When the local concentration of the SCF-mediated c-Kit receptor reaches a certain value, the adjacent D4s will overcome the electrostatic repulsion and combine into the D4-D4 form. The transverse D4-D4 and D5-D5 interaction may accumulate and stabilize the two adjacent membrane proximal external regions of the c-Kit extra-cellular domain. A consequence of the SCF-induced conformational change of the c-Kit extracellular domain is that the transmembrane regions of c-Kit move into close proximity of each other (15 Å); this potentially allows protein-protein interactions between the transmembrane regions as well as positioning the intracellular tyrosine kinase domains close to each other to facilitate their activation and subsequent trans-phosphorylation (Figure 4.3).



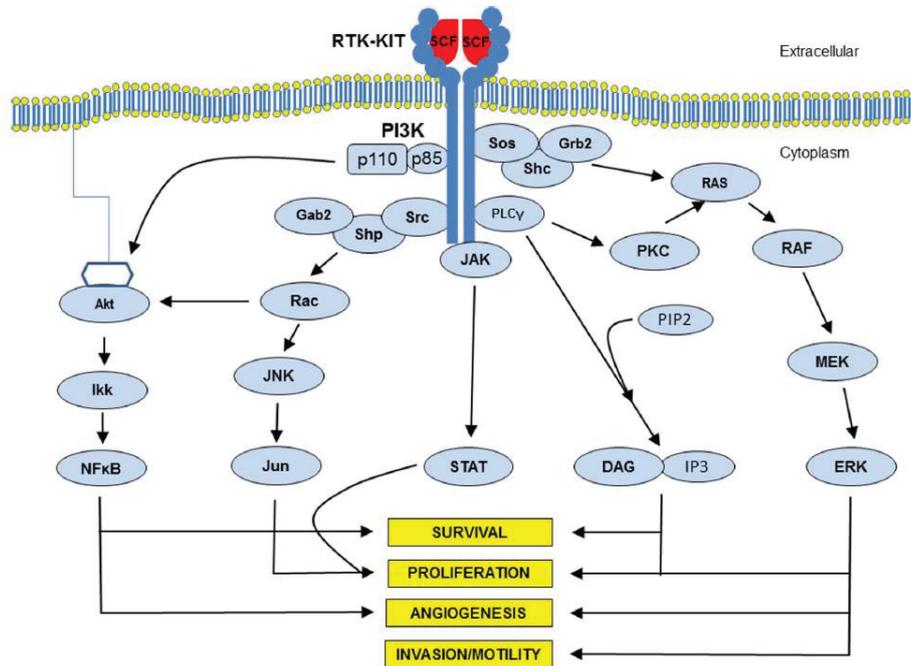
**Figure 4.3** SCF binds and activate c-Kit through three steps: first, SCF integrates with the corresponding domain of c-Kit by electrostatic attraction between SCF and D1-D2-D3 functional unit; second, SCF brings two c-Kit monomers in close proximity to each other, allowing for interactions between D4 and D5 in adjacent c-Kit molecules; third, homodimeric state of c-Kit

caused by the SCF and further stabilized by interactions between D4-D5 allows for efficient trans-phosphorylation in the juxtamembrane region (Tyr568 and 570), kinase insert region (Tyr703, 721, 730, and 747), kinase domain (Tyr823 and 900) (by Src kinases), and COOH-terminal tail (Tyr936) (Lennartsson and Rönnstrand, 2012).

#### **4.4 Downstream signaling pathways of c-Kit**

The downstream signal transduction pathways that are activated include RAS/ERK, PI3K/Akt, phospholipase  $\gamma$ , JAK/STAT and Src kinase pathways (figure 4.4) (Liang et al., 2013; Summy et al., 2006). Activation of the RAS/ERK pathway plays critical roles in cellular proliferation, angiogenesis, cellular migration and invasion through a stepwise activation of RAS, RAF, Mek1/ Mek2 and finally ERK1/ERK2 (Liang et al., 2013; Yasuda and Kurosaki, 2008). The PI3K/Akt pathway promotes cell survival, evasion of apoptosis, angiogenesis, dysregulated cell cycle control and promotion of tumorigenesis, through stepwise activation of I $\kappa$ k and the eventual actions of NF- $\kappa$ B (Liang et al., 2013; Govender and Chetty, 2012). The phospholipase  $\gamma$  pathway, *via* the actions of diacylglycerol and inositol 1,4,5-trisphosphate, promotes cellular proliferation and cell survival (Takahashi and Shibuya, 1997). The interaction of the c-Kit/SCF dimer, with the cytoplasm located JAK tyrosine kinases, results in activation of STAT transcription factors, which then undergo phosphorylation and dimerization. Upon dimerization, the STAT transcription factors enter the nucleus to play integral roles in apoptosis, cellular proliferation and cellular differentiation (Takahashi and Shibuya, 1997). The Src kinase pathway has a complex interaction with the RAS/ ERK, PI3K/Akt and phospholipase  $\gamma$  pathways, with resultant actions of cellular proliferation and angiogenesis (Summy and Gallick et al., 2006; Silva, 2004).

Multiple pathways elicit a negative feedback mechanism on the KIT receptor tyrosine kinase pathway. The E3 ubiquitin-protein ligase Cbl aids in proteosomal degradation of the active KIT tyrosine receptor kinase (Zeng et al., 2005). SH2 domain-containing phosphatase-1 downregulates KIT activity by dephosphorylating the receptor, while negative feedback from protein kinase-C decreases tyrosine kinase activity (Kozlowski et al., 1998; Edling et al., 2007).



**Figure 4.4** The KIT/SCF pathway: cellular interactions. Akt, RAC- $\alpha$  serine/threonine-protein kinase; DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; Gab2, GRB2-associated-binding protein 2; Grb2, growth factor receptor-bound protein 2; Ikk, inhibitor of kappa B kinase; IP3, inositol 1,4,5-trisphosphate; JAK, Janus kinase; JNK, c-Jun N-terminal kinases; Jun, proto-oncogene c-jun; MEK, mitogen/extracellular signal-regulated kinase; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K p85, phosphatidylinositol-4,5-bisphosphate 3-kinase (p85 subunit); PI3K p110, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit  $\alpha$  (p110 subunit); PIP2, phosphatidylinositol biphosphate; PKC, protein kinase C; PLC-  $\gamma$ , phospholipase C-  $\gamma$  Rac, RAS-related C3 botulinum toxin substrate 1; RAS, rat sarcoma viral oncogene homologue; RTK-KIT, receptor tyrosine kinase v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue; SCF, stem cell factor; Shc, SHC-transforming protein 1; Shp, small heterodimer partner; Sos, son of sevenless homologue 1; Src, sarcoma tyrosine-protein kinase; STAT, signal transducer and activator of transcription (Roberts and Govender, 2015).

#### 4.5 The expression pattern and function of c-Kit and SCF in normal physiological condition

The expression pattern of c-Kit and SCF during mouse embryogenesis suggests that they are involved in migration of hematopoietic cell, germ cell, and melanoblast lineages cells (Keshet et al., 1991; Matsui et al., 1990; Orr-Urtreger et al., 1990). Expression data also suggest that c-Kit signaling may have important roles in the nervous system, placenta, heart, lung, and midgestational kidney. Using a transgenic mice model expressing LacZ at the KIT locus, Bernex F et al. (1996) studied not only the importance of c-Kit for the

development of various embryonal structures, but also where c-Kit is normally expressed during embryogenesis. Interstitial cells of Cajal express c-Kit during embryogenesis, while they are not dependent on c-Kit for their function during embryogenesis. c-Kit expression has also been found at endothelial, epithelial, and endocrine cells.

Numerous loss-of-function mutations in *W* and the *Sl* loci have been described in mice. These loci encode c-Kit and SCF, respectively. These naturally occurring mutations comprise a spectrum of defects of c-Kit signaling ranging from minor defects in its tyrosine kinase activity to a complete loss of its kinase activity, resulting in the corresponding degree of severity in the phenotype displayed by these mice (Lev et al., 1994). These mutations have given us hints on the normal physiological function of c-Kit during embryogenesis and adulthood. The vast number of various loss-of function mutations in this receptor/ligand system suggests crucial functions in the hematopoietic system, during gametocyte development, pigmentation, intestinal motility, as well as in the nervous system (Keshet et al., 1991; Russell, 1979). Data from other models have also suggested a function in the immune system including inflammation (given its expression in both dendritic cells and mast cells) and in the regulation of vasculogenesis (Heissig et al., 2003; Ray et al., 2010).

c-Kit has various roles in physiological condition. Here are the major physiological functions of c-Kit briefly described separately:

**Hematopoiesis:** Hematopoietic stem cells (HSC) are characterized by their ability to self-renew and to be able to differentiate into all hematopoietic lineages. The process of lineage commitment results in a diminished ability to self-renew, while their proliferative capacity is increased, which led to expansion in cell numbers. With few exceptions, c-Kit can be detected in early hematopoietic cells (including stem cells and progenitor cells), and its expression is lost during their differentiation (Broudy, 1997; Okada et al., 1991). Early hematopoietic cells are dependent on c-Kit-mediated signals for their proliferation and survival.

**Pigmentation:** The phenotypic observation that mice defective in c-Kit function have defective pigmentation is believed to be linked to the role of c-Kit in proliferation, survival, and haptotactic migration of melanocytes from the neural crest to epidermis during embryogenesis (Mackenzie et al., 1997; Wehrle-Haller, 2003). The exact pathways linking

c-Kit to pigment production are not fully known. The transcriptional factor MITF might play an important role in c-Kit mediated melanocyte action.

**Gastrointestinal:** In humans suffering from a loss-of-function mutation of c-Kit, so-called piebaldism, one of the symptoms is constipation (Giebel and Spritz, 1991) due to loss of the interstitial cells of Cajal (ICC). These are c-Kit positive cells that govern the motility of the gut. The ICC regulate the gut movement by their ability to communicate with both nerve cells and smooth muscle cells. ICC express c-Kit, and in mice with loss-of-function mutations of the c-Kit system, there is a constipation phenotype that suggests an important role of c-Kit in ICC development and function (Hulzinga et al., 1995; Ward et al., 1994).

**Reproduction:** Studies suggest that c-Kit is involved in processes of oogenesis, folliculogenesis, and spermatogenesis. The function of c-Kit in germ cells is strictly dependent on the ability to activate PI3-kinase. Knock-in mouse studies where mice carried a mutant c-Kit (Y719F, the binding site for the p85 subunit of PI3-kinase in mice) resulted in defective PI3- kinase activation and male sterility (Blume-Jensen et al., 2000; Kissel et al., 2000).

**Neuron system:** Studies on mice carrying loss-of-function mutations in either c-Kit or SCF have established a role for c-Kit signaling in the spatial learning function of the hippocampal region of the brain (Katafuchi et al., 2000). Furthermore, c-Kit has been found to be expressed in neuro-proliferative zones in the adult brain and in neuronal cultures (Jin et al., 2002). Administration of SCF in animals leads to proliferation of primitive neurons. c-Kit plays an important role in the migration of neuronal stem- and progenitor cells to sites of injury in the brain (Sun et al., 2004).

**Lung:** Recent data suggest that c-Kit has an important function in maintaining the integrity of lung tissue. It could be shown that mice deficient in c-Kit signaling spontaneously developed airspace enlargement and that static lung compliance increased significantly over time in such mice (Lindsey, 2011). Residual volume and *ex vivo* lung compliance is also significantly increased in mice deficient in c-Kit signaling.

#### **4.6 c-Kit and cancer**

c-Kit has been implicated in numerous human malignancies, including small cell lung carcinoma, malignant melanomas, colorectal cancer, and in more than 80% of cases of gastrointestinal stromal tumors. There are different types of deregulation of c-Kit, and each

of them can result in tumorigenesis. Deregulation of c-Kit can result in cancer in different ways. This deregulation could occur by gain of function, loss of function, overexpression, and point mutations (Huang et al., 1992).

#### **4.6.1 c-Kit expression in cancer**

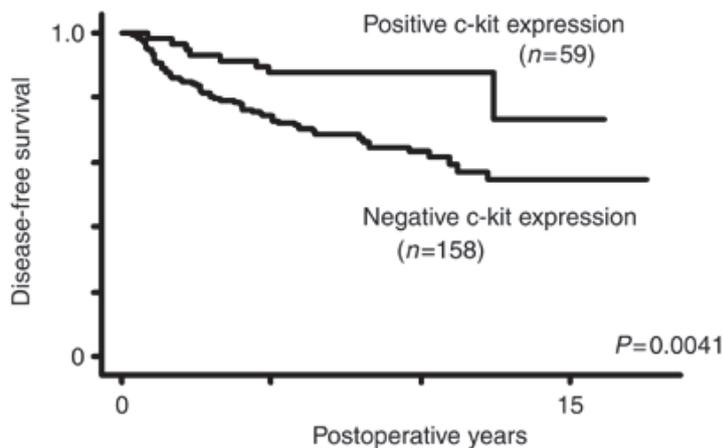
As a classic receptor tyrosine kinase (RTK), c-Kit has been found to be related to numerous kinds of cancers. Upregulation of c-Kit is the cause of several diseases, such as mastocytosis, acute myeloid leukemia (AML) and renal carcinoma. For example, in normal physiological circumstances, only a minority of hematopoietic cells express c-Kit after differentiation, while AML cells express c-Kit, which influences the malignant phenotype of this cancer (Yavuz et al., 2002). A previous study reported that c-Kit expression level is 7.4-fold higher in renal oncocytoma and chromophobe renal carcinoma than that in renal normal tissues (Morgan et al., 1990). However, the evidences showing a loss of c-Kit expression in some cancers, such as breast cancer, neuroblastoma or melanoma, support our hypothesis indicating c-Kit as a new member of the growing dependence receptor family.

In the case of breast cancer, there have been several studies regarding the c-Kit expression in this malignancy, showing that the c-Kit expression decreases in breast cancer tissue comparing to normal epithelium of the mammary gland (Natali et al, 1992a; Matsuda et al, 1993; Hines et al, 1995; Chui et al, 1996; Palmu et al, 2002; Tsuura et al, 2002; Ko et al, 2003; Simon et al, 2004; Ulivi et al, 2004; Yared et al, 2004; Tsutsui et al, 2006; Tramm et al, 2016). The rate of positive c-Kit expression in breast cancer varies from 1 to 82%, which likely attributes to different methods for evaluating c-Kit expression. By contrast, the rate of positive c-Kit expression in the normal breast tissue is 100% in all four studies (Natali et al, 1992; Tsuura et al, 2002; Ulivi et al, 2004; Yared et al, 2004) (Table 4.1). Tsutsui et al. (2006) also indicated that breast cancer patients with negative c-Kit expression tend to have a significantly worse disease-free survival than patients with positive c-Kit expression (Figure 4.5).

Author (year)	Method	c-kit expression				
		Normal breast tissue	Benign tumours	<i>In situ</i> cancers	Invasive primary cancers	Metastatic breast cancers
Natali et al (1992a, 1992b)	IHC <sup>a</sup>	6/6 (100%)	28/36 (78%)		10/80 (13%)	1/40 (3%)
Matsuda et al (1993)	IHC				2/10 (20%)	
Hines et al (1995)	mRNA IHC				9/11 (82%)	
Chui et al (1996)	IHC (IRS) <sup>b</sup>	6.22+2.11 (n=20)	3.33+2.44 (n=58)		0.43+1.27 (n=57)	
Tsuura et al (2002)	IHC	338/338 (100%)	131/141 (93%)	0/11 (0%)	2/171 (1%)	
Palmu et al (2002)	IHC				33/40 <sup>c</sup> (82%)	
Ko et al (2003)	IHC (IRS)	5.90+1.37 (n=20)	4.05+1.82 (n=20)		0.90+1.79 (n=40)	1.06+1.86 <sup>d</sup> (n=18) 0.20+0.63 <sup>e</sup> (n=10)
Yared et al (2004)	IHC	21/21 (100%)	16/24 (88%)	3/29 (10%)	4/41 (10%)	0/4 (0%)
Ulivi et al (2004)	IHC	14/14 (100%)		7/16 (44%)	7/75 (9%)	
	mRNA	14/14 (100%)		12/16 (75%)	3/14 (21%)	
Simon et al (2004)	IHC				43/1654 (2.6%)	

**Table 4.1:** Published series regarding the c-kit expression in breast tissue (Tsutsui et al, 2006).

<sup>a</sup>Immunohistochemistry, <sup>b</sup>Immunoreactive score, <sup>c</sup>All patients have progressive metastatic breast cancer, <sup>d</sup>Average in the metastatic lymph nodes, <sup>e</sup>Average in distant metastases.



**Figure 4.5** Disease-free survival curve stratified according to the c-kit expression in breast cancer patients (Tsutsui et al, 2006).

The second case is melanoma. As mentioned, a lot of research has implicated aberrant KIT signaling in the development and progression of melanoma. However, several studies based on immunohistochemical evaluation have shown that c-Kit is expressed in normal melanocytes and benign nevi, while it is lost with progression to invasive and metastatic forms (Montone et al., 1997; Shen et al., 2003; Zhu and Fitzpatrick, 2006). Consistent with these data, c-Kit expression is lost in a great proportion of melanoma-derived cell lines (Lassam and Bickford, 1992; Natali et al., 1992b; Zakut et al., 1993), and lack of c-Kit expression correlates with a higher metastatic potential of melanoma xenografts in nude mice (Gutman et al., 1994). Furthermore, forced c-Kit expression in c-Kit-deficient melanoma cell lines retards the growth of these cells in nude mice and confers susceptibility to growth arrest and apoptosis in vitro (Huang et al., 1996). Recent study has shown that KIT gene is frequently hypermethylated and silenced in melanoma (Dahl et al., 2015), and this frequent epigenetic silencing of KIT owing to promoter hypermethylation more directly implicates the loss of KIT in melanoma pathogenesis, substantiating suggestions that c-Kit may have a tumor-suppressive function in some types of melanoma such as cutaneous melanoma.

Besides breast cancer and melanoma, similar findings, indicating that c-Kit is involved in the growth and maintenance of the normal epithelium and that its function may be lost following malignant transformation, have also been demonstrated in thyroid (Natali et al., 1995; Franceschi et al., 2017) and renal (Miliaras et al., 2004) cancers.

As mentioned above, the relation between c-Kit expression and disease-free survival in breast cancer patients indicate that c-Kit plays some roles different from receptor tyrosine kinase. Similarly, two other studies on ovarian cancers (Tonary et al., 2000) and neuroblastomas (Krams et al., 2004) also show an association between a loss of the c-Kit expression and poor prognosis. In ovarian cancers, not only the loss of c-Kit expression is associated with a poor prognosis, but also the c-Kit expression level tends to decrease at an advanced stage (Tonary et al., 2000).

#### **4.6.2 c-Kit mutations in cancer**

In cancers, mutations in the c-Kit gene are commonly seen. More than 500 different mutations of c-Kit have been described in human tumors (Sanger Institute Catalogue of Somatic Mutations in Cancer; <http://www.sanger.ac.uk/genetics/CGP/cosmic/>) (Figure 4.6). D816X is the most cited mutation in cancer. However, most of these mutations are

likely to be passenger mutations rather than driver mutations. One can assume that those mutations that are very rarely found are more likely to be passenger mutations, while those repeatedly found are more likely to be driver mutations.

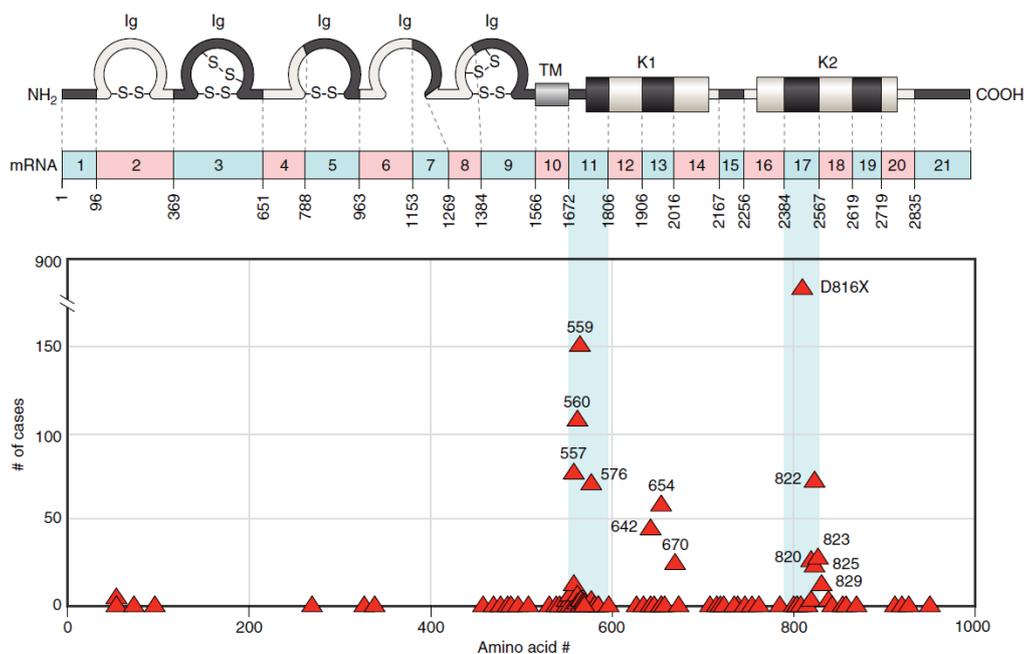
Some “hotspots” in the Kit gene are regular in certain main domain structures (Lanternier et al., 2008). Mutations in domains, such as intracellular and extracellular juxtamembranes, located on exons 8, 9, and 11,12 as well as exon 17, which corresponds to the activation loop in the kinase domain (Schnittger et al., 2006), disrupt the autoinhibitory mechanisms of c-Kit (Sakuma et al., 2003; Orfao et al., 2007). The importance of these two domains is reflected in their critical role in the c-Kit structure and function. The mutation of those two domains frequently causes c-Kit constitutively kinase active. For example, the D816V mutation causes a local structural alteration of the activation loop (A-loop), and a long-range structural re-organization of the juxta-membrane region (JMR) followed by a weakening of the interaction network with the kinase domain, leading D816V-mutated c-Kit constitutively active (Laine et al., 2011). Moreover, the structure and kinase active state change caused by D816V mutation result in imatinib (Gleevec) unable to bind to the ATP binding domain of c-Kit. Thus, D816V mutated c-Kit is resistant against imatinib treatment (Mol et al., 2004). The KIT cDNA structure in different cancers and their respective mutations is illustrated in Figure 4.7.

The first group of extracellular juxtamembrane domains is responsible for the correct binding of the receptor monomer and the stabilization of dimers, which contain dimeric SCF (Nakata et al., 1995). The mutations in this region, particularly in exons 8 and 9, are detected in AML and GIST, respectively (Kimura et al., 1997; Scheijen and Griffin, 2002).

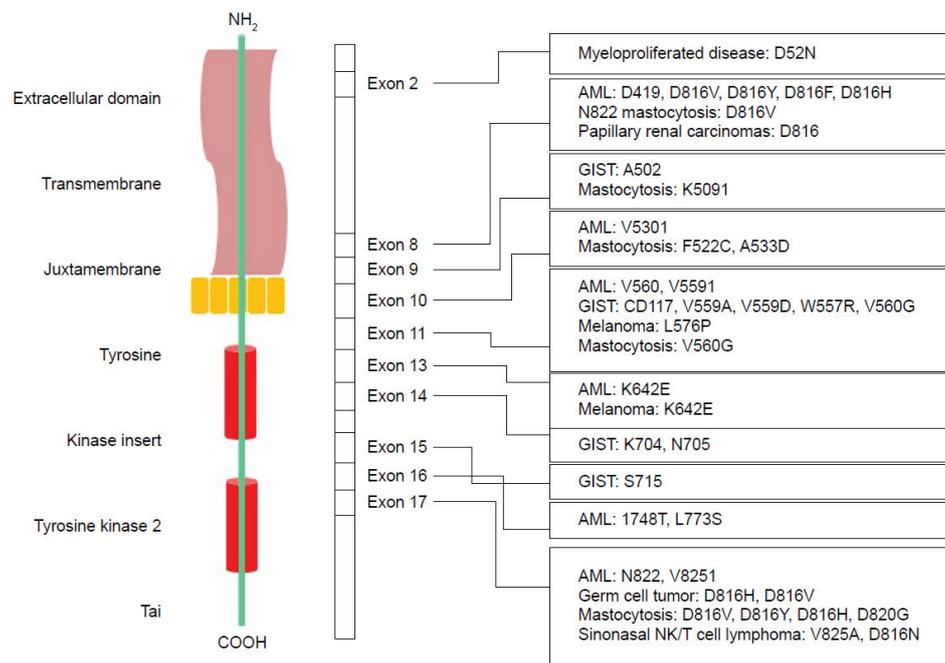
c-Kit mutations occur within exon 11 in almost 65% of all GIST (Gastrointestinal stromal tumor) cases. This exon encodes a key autoregulatory domain of the RTK, which is the intracellular juxtamembrane domain, and stabilizes the inactive conformation of the kinase domain (Heinrich et al., 2000). In addition, mutations in other exons, such as exons 11 and 17, have been identified in GIST and hematological cancers, respectively.

The most detected c-Kit mutations that lead to melanoma are located within exons 11 and 13, namely, L576P and K642E, respectively (Lin et al., 2004). Mutations in exon 17, which encodes the activation loop of the kinase domain, result in hemopoietic malignancies in germ cell tumors. After kinase activation, conformational shifts occur in this region (Orfao et al., 2007).

In a number of cancers, c-Kit activation was detected through overexpression or mutations. Conversely, in other tumors, such as melanoma (Hongyo et al., 2000; Büttner et al., 1998), thyroid carcinoma (Ashman et al., 1999), and breast cancer (Ashman et al., 1999), loss-of-function mutation of c-Kit was observed. Moreover, c-Kit gain-of-function mutation in metastatic melanoma induces apoptosis (Beghini et al., 2000). By contrast, in uveal melanomas, c-Kit expression results in cell proliferation, for which treatment with kinase inhibitor drugs leads to apoptosis induction (Tian et al., 1999). The activating mutation of c-Kit, namely, L576P, has been reported in a small subset of highly metastatic melanomas (Pauls et al., 2004). Thus, there are various c-Kit mutations involved in melanoma, so melanoma can be used as a model to clarify the complex roles of c-Kit in tumorigenesis.



**Figure 4.6** The relative abundance of various c-Kit mutations. Exons 11 and 17 show up as hotspots for mutation. In this way of quantitation, D816X mutations show up as the most common mutation (Johan Lennartsson and Lars Rönstrand, 2012).



**Figure 4.7** KIT cDNA and protein structure in different cancers and their respective mutations (Babaei et al., 2016).

#### 4.6.3 Therapeutic treatment of c-Kit related diseases

Cancer is a major disease threatening human health and the whole world has been paying close attention to its treatment. Traditional chemotherapy is not completely effective for much tumor treatment because of their nonspecific blocking tumor cells division and damage to normal cells. Also, the GIST patients with complete resection still have a very high risk of recurrent. The appearance and development of c-Kit antibodies, especially the monoclonal antibodies, promote the research of c-Kit receptor for continuous activation mechanism. It is generally believed that the dimerization of c-Kit receptor is closely related to its continuous activation and thereby it is considered to be c-Kit receptor-related tumor pathogenesis. With in-depth broadband research to c-Kit receptor, therefore, molecular targeted therapy due to its specificity and low toxicity is increasingly becoming a hot spot.

**Imatinib:** Small molecule tyrosine kinase inhibitors (TKIs) are orally active compounds, many of which are able to compete with the ATP-binding site of the catalytic domain of oncogenic TKs. Imatinib mesylate (Gleevec®) is one of the first examples of the

successful solid tumor treatment TKIs drug in humans, which is first designed for chronic myelogenous leukemia. It can inhibit enzymatic activity of several tyrosine kinases including c-Kit and the platelet-derived growth factor receptor. Later imatinib was gradually applied to treat GIST with good results (Heinrich et al., 2017). GIST patients treated with imatinib had a control rate of 80% to 90%, most showed partial remission, 12% showed complete remission. The sensitivity of imatinib is closely related to c-Kit receptor gene. The action of imatinib in the treatment of GIST has been extensively evaluated and it is currently the first line treatment for metastatic disease and in an adjuvant setting for prevention of relapse of poor prognosis GIST following surgical resection.

Imatinib has been demonstrated to be able to inhibit c-Kit mutated mucosal melanoma cells proliferation with induced apoptosis and reduced downstream signaling regulatory molecules (p42/44, AKT, MTOR, STAT1, STAT3, P70S6K, S6K, etc.) but the wild-type had no changes (Jiang et al., 2008).

At an early stage of evaluation, it was noted that clinically relevant KIT mutants differed greatly in their sensitivity to imatinib. Specifically, substitutions at position D816 in the activation loop rendered the kinase almost completely resistant to the drug at clinically achievable doses (Frost et al., 2002; Ma et al., 2002). This likely reflects the mutation strongly favoring the active conformation of c-Kit kinase domain to which, as in the case of BCR/ABL, imatinib is not able to bind (Mol et al., 2004; Schindler et al., 2000) In contrast, an exon 11 mutation resulting in the substitution V560G enhanced sensitivity to imatinib by more than 10-fold (Frost et al., 2002).

Imatinib has also been evaluated for treatment of systemic mastocytosis (SM) which is typically characterized by activation loop mutations in KIT, particularly the D816V substitution (Valent et al., 2005). As stated, this mutant form of KIT is highly resistant to the drug and most cases have proved refractory. Early trials of imatinib in unselected cases of AML were unsuccessful (Piccaluga et al., 2007) probably due to the relatively low rate of KIT mutations (around 7% overall) and the frequency of resistant D816 mutations.

Unfortunately, even for the patients treated with Imatinib, most of patients will gradually develop resistance to imatinib. 9% to 14% of GIST patients treated with imatinib in the first three months will have an early resistance; approximately half of the patients with metastatic disease develop resistance within 2 years (Corless et al., 2011). The resistance

mechanism may be ascribed to the occurrence of c-Kit gene secondary mutations (Debiec-Rychter et al., 2006), c-Kit gene amplification in a large number, the absence of wild-type allele, or lack of blood concentration of imatinib (Liang et al., 2013). Because of resistance to imatinib, a novel and effective inhibitor with no/little resistance has to be explored.

**Other inhibitors targeting c-Kit:** The critical shortcoming of imatinib prompted the design of novel TKIs with improved selectivity against specific c-Kit mutants for the treatment of resistant forms of GISTs, but also for other diseases associated with c-Kit activation. Sunitinib (Sutent, Pfizer, New York, NY, USA), introduced as an antiangiogenic drug owing to its inhibition of VEGF receptors, proved effective in imatinib-resistant patients with advanced GIST (Demetri et al., 2006), and is now its second-line approved treatment. Dasatinib (Sprycel, Bristol-Myers Squibb, New York, NY, USA) (Shah et al., 2006), have been shown to inhibit D816V mutants. Table 4.2 shows the list of c-Kit inhibitors (including Imatinib) and their other targets, while Table 4.3 shows the list of c-Kit inhibitors based on the diseases that they are tested on.

Inhibitor Name	c-Kit	Other Targets
<u>Dasatinib</u>	++	Abl,Src
<u>Imatinib Mesylate (STI571)</u>	+	PDGFR,v-Abl
<u>Axitinib</u>	++++	VEGFR1/FLT1,VEGFR2/Fik1,VEGFR3
<u>Pazopanib HCl (GW786034 HCl)</u>	+	VEGFR1,VEGFR2,VEGFR3
<u>Dovitinib (TKI-258, CHIR-258)</u>	++++	FLT3,VEGFR3/FLT4,FGFR1
<u>Masitinib (AB1010)</u>	+	Lyn B,PDGFR $\alpha$ ,PDGFR $\beta$
<u>Tivozanib (AV-951)</u>	++	VEGFR2,VEGFR3,EphB2
<u>Amuvatinib (MP-470)</u>	+++	PDGFR $\alpha$ (V561D),FLT3 (D835Y)
<u>Motesanib Diphosphate (AMG-706)</u>	+++	VEGFR1,VEGFR2,VEGFR3
<u>OSI-930</u>	+	FLT1,Kdependence receptor,CSF-1R
<u>Ki8751</u>	++	VEGFR2,PDGFR $\alpha$
<u>Telatinib</u>	++++	VEGFR3,VEGFR2,PDGFR $\alpha$
<u>Pazopanib</u>	++	VEGFR1,VEGFR2,VEGFR3
<u>Dovitinib (TKI-258) Dilactic Acid</u>	++++	FLT3,FGFR1,VEGFR3/FLT4
<u>Tyrphostin AG 1296</u>	+	PDGFR,FGFR (Swiss 3T3)
<u>Sitravatinib (MGCD516)</u>	+++	Ddependence receptor2,EPHA3,Axl
<u>Pexidartinib (PLX3397)</u>	+++	CSF-1R,Flt3
<u>Dasatinib Monohydrate</u>	++	Abl ,Src
<u>Dovitinib (TKI258) Lactate</u>	++++	FLT3,VEGFR3/FLT4,FGFR1
<u>AZD2932</u>	+++	PDGFR $\beta$ ,Flt3,VEGFR-2
<u>Sunitinib Malate</u>	√	FLT3,PDGFR $\beta$ ,VEGFR2
<u>Sunitinib</u>	√	FLT3 ,PDGFR $\beta$ ,VEGFR2

**Table 4.2** List of c-Kit inhibitors

Notes: “+” refers to an inhibitor that has a significant effect on the specific signaling target. If the IC50 of the minor target of any inhibitor is 1,000 times greater than the IC50 of the major target, its minor target will not be mentioned in any table. Adapted from Selleckchem.com, <http://www.selleckchem.com/c-Kit.html>. Abbreviation: IC50, half-maximal inhibitory concentration.

Name	Targets	IC <sub>50</sub> <sup>a</sup> (nM)	Formula	Molecular weight (g/mol)	FDA-approved inhibitor	Clinical trial information testing on
Amuvatinib (MP-470)	c-Kit, PDGFR $\alpha$ , Flt3	10, 40, 81	C <sub>23</sub> H <sub>21</sub> N <sub>5</sub> O <sub>3</sub> S	447.51	Approved by the FDA for CML, GISTs and a number of other malignancies	Lymphoma, unspecified adult solid tumor, solid tumors, malignant disease, small-cell lung carcinoma
Axitinib	VEGFR1, VEGFR2, VEGFR3, PDGFR $\beta$ , c-Kit	0.1, 0.2, 0.1–0.3, 1.6, 1.7	C <sub>22</sub> H <sub>18</sub> N <sub>4</sub> O <sub>5</sub>	386.47	Approved by the FDA	Advanced renal cell carcinoma, renal cell carcinoma, nonclear cell, temsirolimus-resistant renal cell carcinoma, pheochromocytoma, paraganglioma, advanced solid tumors
Cabozantinib (XL184, BMS-907351)	VEGFR2, c-Met, Ret, Kit, Flt-1/3/4, Tie2, AXL	0.035, 1.3, 4, 4.6, 12/11.3/6, 14.3, 7	C <sub>28</sub> H <sub>24</sub> FN <sub>3</sub> O <sub>5</sub>	501.51	Approved by the FDA for renal cell carcinoma	Medullary thyroid cancer, prostate cancer, castration-resistant prostate cancer, prostatic neoplasms, colorectal cancer, uterine sarcoma, and prostate cancer
Dasatinib	Abl, Src, c-Kit	1, 0.8, 79	C <sub>22</sub> H <sub>26</sub> ClN <sub>7</sub> O <sub>2</sub> S	488.01	Approved by the FDA for Ph+ CML	AML, breast cancer, recurrent childhood brain tumor, lung cancer/NSCLC, chronic myeloid leukemia
Dovitinib (TKI-258, CHIR-258)	FLT3, c-Kit, FGFR1/3, VEGFR1-4, InsR, EGFR, c-Met, EphA2, Tie2, IGF-1R, and HER2	1/2, 8–13	C <sub>21</sub> H <sub>21</sub> FN <sub>6</sub> O	392.43		

**Table 4.3** c-Kit inhibitors classification based on their targets, chemical and structure formulae, and diseases they are tested on.

Notes: Data from Selleckchem.com, <http://www.selleckchem.com/c-Kit.html#####>.

The IC<sub>50</sub> is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.

Abbreviations: IC<sub>50</sub>, half-maximal inhibitory concentration; FDA, Food and Drug Administration; CML, chronic myelogenous leukemia; GIST, gastrointestinal stromal tumor; AML, acute myeloid leukemia; NSCLC, non-small-cell lung cancer.

## II. Aims of Thesis Project

Dependence receptors represent a new family of cellular receptor, able to initiate two opposite signaling pathways, respecting the presence of their ligand. In the few past year, several receptors has been recognized to belong to the functional family of dependence receptor, most of them originally presenting pro-survival features, such as the tyrosine kinase receptors TrkA and TrkC (Nikoletopoulou et al., 2010; Tauszig-Delamasure et al., 2007), Plexin D1 (Luchino et al., 2013), Notch3 (Lin et al., 2017) and Kremen1 (Causeret et al., 2016). Interestingly, several of these new dependence receptors was not previously described to induce apoptosis neither to act as tumor suppressors. Thus, increasing the member of the dependence receptor family will allow us to better understand the relation between tumor escape and cell death, as well as to develop innovative therapeutic anti-cancer strategies.

For this purpose, in this work, we focused on the proto-oncogene c-Kit, a type III-receptor tyrosine kinase (RTK), a class of membrane receptors playing a crucial role in cell proliferation, differentiation and migration through different signaling pathways (Ali and Ali, 2007). This receptor is often overexpressed or mutated in numerous human malignancies. However, in some tumors, such as neuroblastoma, invasive melanoma, thyroid carcinoma and breast cancer (Krams et al., 2004; Natali et al., 1995; Natali et al., 1992a; Natali et al., 1992b), c-Kit expression seems to be lost during tumor progression and related to disease progression. Moreover, removal of SCF or addition of a monoclonal c-Kit antibody trigger apoptosis in murine melanocyte precursors (Ito et al., 1999). These behaviors are not proper to an oncogene, but are rather similar to the comportment of a dependence receptor, whose expression is often lost in cancer and leads to tumor progression and metastatic dissemination. Moreover, some of dependence receptor family members are receptor tyrosine kinases, such as TrkC (Tauszig-Delamasure et al., 2007), EphA4 (Furne et al., 2009), MET (Tulasne et al., 2004) and RET (Bordeaux et al., 2000). These evidences led us to formulate the hypothesis that c-Kit could belongs to the dependence receptor family.

To validate this hypothesis we will generate several inducible stable cell lines, in order to express c-Kit following doxycycline treatment. To demonstrate that c-Kit belongs to the dependence receptor family, we will (i) assess its ability to induce cell death in the absence of its ligand, (ii) evaluate whether its ligand SCF is able to revert cell death induction, (iii) verify if c-Kit could be a caspase substrate and (iv) validate whether c-Kit could act as a tumor suppressor. Moreover, we will also (v) decipher the molecular mechanisms

eventually involved in c-Kit-induced cell death and (vi) prove that silencing c-Kit ligand or interfering with the binding between SCF and c-Kit could induce cell death in cellular models expressing SCF.

This information could have a vast impact on the development of an innovative anticancer strategy. Indeed, including c-Kit in the emerging family of dependence receptors could allow the treatment of SCF-expressing tumours, which until now could be targeted only by inhibiting kinase activity, an approach raising tumour resistance. Indeed, we propose that this kind of tumors could also be treated by exposing the putative pro-apoptotic activity of c-Kit. This could be achieved by interfering with the binding between c-Kit and SCF, using decoy peptides or neutralizing monoclonal antibodies.

# III. Results

**5. Article: The proto-oncogene c-Kit inhibits tumor growth by  
behaving as a dependence receptor**

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# The proto-oncogene c-Kit inhibits tumor growth by behaving as a dependence receptor

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## Summary

c-Kit is a classic proto-oncogene either mutated or up-regulated in cancer cells and this leads to its constitutive kinase activation and thus to uncontrolled proliferation. While the pro-oncogenic role of c-Kit is of no doubt, some observations do not fit well with c-Kit solely as a tumor-promoting moiety. We show here that c-Kit is actively triggering cell death in various cancer cell lines unless engaged by its ligand Stem Cell Factor (SCF). This pro-death activity is enhanced when the kinase activation of c-Kit is silenced and is due to the c-Kit intracellular cleavage by caspase-like protease at D816. Moreover, in vivo, over-expression of a c-Kit kinase dead mutant inhibits tumor growth and this intrinsic c-Kit tumor suppressive activity is

dependent on the D816 cleavage. Thus, c-Kit is acting both as a proto-oncogene via its kinase activity and as a tumor suppressor via its dependence receptor activity.

## Introduction

Evasion of cell death is a hallmark of cancer (Hanahan and Weinberg, 2000), both for tumor transformation and resistance to anticancer agents (Hersey and Zhang, 2003). For this reason, cell death mechanisms and the re-engagement of cell death in cancer cells have been investigated in details over the last past twenty years. While most of the literature and pharmaceutical efforts have been focused on both the extrinsic and intrinsic pathway for apoptosis (Meier and Vousden, 2007), over the past few years, an alternative pathway for cell death and its associated tumor suppressive activity have emerged. This cell death pathway is induced by a functional family of membrane receptors, dubbed dependence receptors. These receptors, despite their ability to transduce intracellular signals in the presence of their ligand, are also active in the absence of their ligand and in these settings they trigger apoptosis induction (Gibert and Mehlen, 2015; Paradisi and Mehlen, 2010). Expression of such dependence receptor renders cells dependent on availability of the ligand for survival (Mehlen and Puisieux, 2006). To date, more than 20 receptors has been identified as dependence receptors (Gibert and Mehlen, 2015), sharing the ability to induce cell death in the absence of their respective ligand. This includes the netrin-1 receptors DCC and UNC5H (Llambi et al., 2001; Mehlen et al., 1998), Sonic Hedgehog receptors Patched and CDON (Delloye-Bourgeois et al., 2013; Thibert et al., 2003) or more recently Kremen1 (Causeret et al., 2016) or Notch3 (Lin et al., 2017). Because of this pro-death activity, most of them have been hypothesized to

act as tumor suppressors, being down-regulated in cancers (Bernet et al., 2007; Fearon et al., 1990; Shin et al., 2007; Thiebault et al., 2003). Along this line silencing of the netrin-1 receptor DCC, the SHH receptor CDON or the specific inactivation of the pro-death activity of DCC is associated with cancer progression in mice (Bernet et al., 2007; Castets et al., 2011; Delloye-Bourgeois et al., 2013; Krimpenfort et al., 2012). Of interest the receptors tyrosine kinase RET, MET and TrkC were also shown to act as dependence receptors (Bordeaux et al., 2000; Tauszig-Delamasure et al., 2007; Tulasne et al., 2004). We thus investigated whether another receptor tyrosine kinase, c-Kit, shown to be key for tumor progression could be also acting as a dependence receptor.

The proto-oncogene c-Kit, also known as CD117, was initially identified as a viral oncogene in the Hardy-Zuckerman IV feline sarcoma virus (HZ4-FeSV) and associated with its transforming activity (Besmer et al., 1986). It codes for a type III receptor tyrosine kinase (RTK), a class of membrane receptors playing a crucial role in cell proliferation, differentiation and migration through different signaling pathways (Ali and Ali, 2007). Signaling of c-Kit is activated by the binding of its ligand, the Stem Cell Factor (SCF) protein (Zsebo et al., 1990), causing c-Kit dimerization and activation by autophosphorylation and initiating a phosphorylation cascade resulting in the regulation of cell growth (Sattler and Salgia, 2004). Several studies have been highlighted the major role of the SCF/c-Kit system in melanogenesis, gametogenesis and hematopoiesis (Lennartsson et al., 2005), as well as in brain angiogenesis (Sun et al., 2006). Moreover, dysregulation of c-Kit signaling or gain-of-function mutations are correlated with tumorigenesis, in particular in acute myeloid leukemia (AML), gastrointestinal stromal tumors (GIST) and mastocytosis (Lennartsson et al., 2005). Gain-of-function somatic

mutations trigger a constitutive activation of c-Kit without SCF binding, leading to tumorigenesis. Moreover, expression of SCF and c-Kit has been reported in breast tumor cells, small cell lung cancer cells, gynecological tumors, and malignant glioma (Lennartsson and Ronnstrand, 2012). In neuroblastic tumors, such as neuroblastoma, the role of c-Kit function is still controversial. While some reports showed no correlation between c-Kit/SCF and clinical characteristics, several groups have recently demonstrated that c-Kit expression is closely associated with differentiation and with a good prognosis (Krams et al., 2004). Indeed, the loss of expression of c-Kit receptor may be related to neuroblastoma disease progression. This trait is not usually expected for an oncogene, but is rather seen with dependence receptors, whose expression is often lost in cancer and lead to tumor progression and metastatic dissemination. We show here that c-Kit receptor belongs to the dependence receptor family, thus it is able to induce apoptotic cell death in the absence of its ligand SCF, which in turn is sufficient to inhibit apoptosis induction. Moreover, we show that c-Kit, despite its well-documented role as oncogene, could have an intrinsic tumor suppressor behavior via its pro-death activity.

## Results

### **c-Kit is intrinsically triggering apoptotic cell death**

To study the effect of c-Kit on cell death, we generated several stable cell lines, in which c-Kit expression is induced by adding doxycycline in the culture medium. To avoid positive signaling (i.e., kinase activation) that may induce survival signals, we generated kinase-mutated c-Kit expressing cell lines, using a c-Kit construct harboring D792N mutation, which was reported to be kinase activity mutated (Roskoski, 2005). Inactivation of c-Kit kinase activity was verified by evaluating c-Kit auto-phosphorylation, as well as ERK1/2 phosphorylation (Figure S1A). Moreover, kinase-inactivating mutation did not affect cellular membrane localization of c-Kit (Figure S1B). Interesting, the inducible system used here, established from sleeping beauty vectors, allowed c-Kit expression at levels comparable to endogenous levels detected in M2G2 and XPC melanoma cell lines (Figure S1C). Forced expression of wild-type (WT) or mutant c-Kit following doxycycline treatment induced a dose-dependent increase of PI-positive cells, compared to untreated cells, in neuroblastoma (IMR32) or melanoma (HMCB) cell lines (Figure 1A and S1DE). In A549 cell line, over-expression of WT c-Kit was not sufficient to induce cell death while the kinase dead c-Kit was massively enhancing cell death. We investigated whether cell death induced by c-Kit over-expression was apoptosis by assessing Annexin V staining rate in IMR32 and HMCB cell lines upon treatment with doxycycline. Over-expression of kinase-mutated c-Kit induced an increase of Annexin V-positive population, compared to untreated cells (Figure 1B).

The implication of apoptosis was further demonstrated by measuring caspase activity. As shown in Figure 1C, over-expression of c-Kit was able to strongly induce caspase activity, compared to untreated IMR32 and HMCB cells, especially kinase-mutated c-Kit. Moreover, c-Kit over-expression was also sufficient to induce caspase-3 cleavage (Figure 1D), a specific hallmark of the apoptotic process. Because apoptosis induction is often associated with Mitochondrial Outer Membrane Permeabilization (MOMP), a critical event governing the release of pro-apoptotic molecules (Kalkavan and Green, 2018; Li et al., 1997), we showed that c-Kit forced expression in HMCB cells induced MOMP (Figure 1E). Finally, the implication of caspase in cell death induced by c-Kit was confirmed by analyzing cell death upon treatment with pan-caspase inhibitor Q-VD-OPh. Caspases inhibition completely suppressed DNA fragmentation induced by kinase-mutated c-Kit over-expression (Figure 2A). Additionally, Q-VD-OPh inhibited the increase of the Annexin V-positive population, induced by kinase-mutated c-Kit over-expression in HMCB cells (Figure S1F). In a similar way, the use of a caspase-3 inhibitor prevented PI-positivity induced by WT or kinase-mutant c-Kit over-expressed in A549 and IMR32 cells (Figure 2B). To further analyze which caspases were involved in c-Kit-induced cell death, we generated stable HMCB cells inducible for c-Kit and knocked-down for caspase-3 and -9, using CRISPR/Cas9 strategy. Knocking-down of caspase-3 or -9 was sufficient to inhibit DNA fragmentation observed upon c-Kit induction in HMBC cells (Figure 2C). Moreover, we observed a similar inhibitory effect on Annexin V-positive population induced by c-Kit over-expression in IMR32 cells (Figure S1GH). Collectively, these data strongly suggest that c-Kit is actively triggering cell death and this death activity is, at least in part, masked by the c-Kit kinase activity.

**c-Kit-induced apoptosis is inhibited by its ligand SCF and is mediated by intracellular caspase-like cleavage driving a caspase activation amplification loop**

We then assessed whether, as expected for a dependence receptor, the ligand of c-Kit could inhibit cell death induction (Goldschneider and Mehlen, 2010). As shown in Figure 3A, while in A549 cell line the induction of kinase-mutated c-Kit strongly increased the number of PI-positive cells, treatment with SCF clearly inhibited cell death induced by doxycycline treatment. Similarly, treatment of IMR32 cells with SCF was sufficient to inhibit caspase-3 activation and cleavage induced by c-Kit over-expression (Figure 3B and S2A), as well as to decrease the percentage of Annexin V-positive cells (Figure S2B). These results show that the ligand SCF is able to revert apoptosis induced by c-Kit.

Caspases appear to be key components in mediating the death of most dependence receptors and most of them are cleaved by caspase in their intracellular domain as a prerequisite for cell death engagement. To assay whether c-Kit could also be a substrate for caspases, we translated *in vitro* the intracellular region of c-Kit and we incubated this domain with purified active caspase. As shown in Figure 3C, this intracellular domain was cleaved *in vitro* by active caspase, generating cleavage products that migrate at molecular masses of 35 and 18 kDa. To identify the putative caspase cleavage site, we systematically mutated aspartic acid residues that could match with the cleavage products. Of interest, the mutation in the intracellular region of Asp-816 to Valine (D816V) abolished cleavage and the generation of the fragments at 35 and 18 kDa (Figure 3C). Moreover, we confirmed c-Kit cleavage by caspases in cells, by generating IMR32 and A549 cell lines stably expressing the intracellular region of c-Kit (Figure 3D and

S2C). In presence of the proteasome inhibitor MG132 -used here to increase the stability of the cleaved fragment-, a fragment migrating at molecular mass of 18 kDa appeared in stable cells expressing WT c-Kit, while this band was not detected when we generated the D816V mutated construct. Similar 18kDa fragment was observed when stable cells expressing full-length c-Kit were treated with Bortezomib, another proteasome inhibitor (Adams et al., 1999) (Figure 3E). In addition, we confirmed the cleavage of endogenous c-Kit, treating melanoma M2Ge cells with Bortezomib or MG132 proteasome inhibitors (Figure 3F and S2D). Moreover, c-Kit caspase cleavage was enhanced by treating M2Ge cells with Bortezomib and caspase activators such as BV6, a dual cIAP and XIAP inhibitor (Li et al., 2011) or PAC1, a potent direct procaspase-3 activator (Putt et al., 2006) (Figure S2E). Thus, together these data support the view that c-Kit is cleaved by caspase-like protease *in vitro* and in cell culture at Asp-816. Of key interest, D816V c-Kit mutation has been extensively reported in the literature as a mutation associated with tumor resistance to Gleevec (Frost et al., 2002).

Another trait shared by several dependence receptors is their ability, once cleaved, to recruit a protein complex encompassing and activating caspase-9 (Delloye-Bourgeois et al., 2013; Forcet et al., 2001; Lin et al., 2017; Mille et al., 2009). Because caspase-9 was shown to be required for c-Kit-induced apoptosis, we investigated whether c-Kit could interact and activate caspase-9. We thus performed a co-immunoprecipitation assay between c-Kit and caspase-9. As shown in Figure 3G, caspase-9 is efficiently pulling down kinase-mutated c-Kit. To investigate further whether c-Kit, when expressed upon doxycycline treatment, could not only interact but could also activate caspase-9, we performed c-Kit pull-down and assessed the

caspase activity contained in the pull-down. As shown in Figure 3H, c-Kit pull down retains a large caspase-9 activity.

To explore the role of caspase cleavage in c-Kit-induced apoptosis, we next generated stable cell lines inducible for double mutated c-Kit (D792N/D816V), thus lacking kinase activity and the possibility to be cleaved by caspases. While the double mutation did not affect cellular membrane localization of c-Kit (Figure S1B), mutation of the putative caspase cleavage site dramatically altered the pro-apoptotic activity of kinase-mutated c-Kit. Indeed, in IMR32 and A549 cell lines, over-expression of double-mutated c-Kit was not anymore able to increase PI-positivity (Figure 4A and S2F), as compared to kinase-mutated c-Kit. Moreover, mutation of the putative caspase site at D816 inhibited the increase of Annexin V-positive population observed after induction of kinase-mutated c-Kit (Figure 4B), as well as caspase-3 cleavage (Figure S2G). In a similar way, D816V mutation affected the ability of c-Kit to promote MOMP (Figure 4C). We then investigated the relationship between caspase cleavage of c-Kit and caspase-9 recruitment/activation. Interesting, caspase-mutated c-Kit was not able to interact with caspase-9 (Figure 4D). Moreover, when performing a pull-down of c-Kit after doxycycline treatment, the caspase-9 activity included in the pull-down was dramatically reduced when double-mutated c-Kit was pulled down instead of kinase-mutated c-Kit (Figure 4E). Collectively, these results show that c-Kit shares the general traits of a dependence receptor, triggering cell death *via* a mechanism requiring cleavage of its intracellular domain at D816, a key amino acid involved in tumor resistance.

#### **c-Kit acts as a tumor suppressor**

Most of the described dependence receptors act as tumor suppressors. We thus hypothesized that c-Kit, known as a classic proto-oncogene because of its kinase activity, could actually intrinsically hold a tumor suppressive activity that is somehow masked by the c-Kit kinase activity. To show this, HMCB cells, inducible for kinase-mutated c-Kit, were engrafted in severe combined immunodeficiency (SCID) mice and animals with tumors were treated daily by i.p. injections of doxycycline (or vehicle), in order to induce c-Kit expression in engrafted tumors (Figure S3AB). While vehicle injection had no effect on tumor growth, expression of kinase dead c-Kit induced a strong and prolonged tumor growth inhibition (Figure 5A). Moreover, final tumor size was significantly lower in the doxycycline-treated tumor group, compared to the control treated group (Figure 5B). Quantification of TUNEL positive cells on the tumor sections from recovered tumors also indicated that kinase-mutated c-Kit was able to trigger more cancer cell death *in vivo* (Figure 5C).

To more formally demonstrate the role of c-Kit pro-apoptotic activity in this tumor suppressive effect, we engrafted HMCB cell line, inducible for kinase dead c-Kit mutated in its caspase cleavage site (D792N/D816V). Mutation of caspase site was sufficient to prevent the tumor suppressor activity of c-Kit, as expression of kinase- and caspase-mutated c-Kit had no more tumor growth inhibiting effect (Figure 5D), despite a similar expression of c-Kit and c-Kit D792N/D816V in both stable cell lines (Figure S3C). In addition, there was no difference between final sizes of tumors obtained in double mutant c-Kit cells and injected with vehicle or doxycycline, while tumors obtained from kinase-mutated c-Kit, harboring a wild-type caspase site, were significantly smaller (Figure 5EF).

### **SCF interference triggers apoptosis and limits tumor growth**

Following this hypothesis, blocking interaction of SCF with c-Kit should both inhibit the kinase activity and unleash the killing activity. We thus investigated whether, in cancer cell settings, the SCF autocrine production extensively reported (Lennartsson and Ronnstrand, 2012; Theou-Anton et al., 2006) could not only activate the c-Kit kinase activity but also block the death activity of endogenous c-Kit. To demonstrate that endogenous c-Kit was able to trigger apoptosis in the absence of SCF, we silenced the ligand by RNA interference strategy in various cancer cells. A549 cells transfected with siRNAs specific for SCF showed a strong increase of caspase-3 activity, compared to cells transfected with a control, non-specific siRNA (Figure 6A and S4A). We found a similar effect by transfecting HCT116 cell line with SCF siRNAs (Figure S4B), expressing very high levels of the ligand. Moreover, SCF silencing enhanced the number of PI-positive cells in transfected A549 cells (Figure 6B). In A549 and HCT116 cells, the endogenous level of c-Kit is relatively low, supporting the view that in these cell lines there is no spontaneous auto-activation of the kinase activity of c-Kit that may interfere with the pro-apoptotic activity of c-Kit. Of interest, in melanoma M2Ge cells, expressing high levels of the receptor and thus showing receptor auto-activation (Figure S1C), silencing of SCF failed to affect cell viability (Figure 6C). However, when in these M2Ge cells we inhibited kinase activity using Imatinib treatment, we observed a massive enhancement of cell death induction upon SCF silencing (Figure 6C).

Next, we investigated a way to interfere with the binding between c-Kit and SCF in a more therapeutically relevant manner than RNA interference. For this purpose, we generated a stable HCT116 cell line, inducible for the expression of the extracellular domain of c-Kit (c-

Kit-EC, Figure 6D), secreted into the extracellular milieu (Figure 6E). This recombinant protein has been shown to interact with SCF (Philo et al., 1996), thus probably acting as a trap able to interfere with the binding between SCF and the membrane-bound c-Kit (Lev et al., 1992). Over-expression of c-Kit-EC upon treatment with doxycycline triggered apoptotic cell death, as shown by induction of a strong increase of the number of PI-positive cells (Figure 6F), Annexin V-positive population (Figure 6G), DNA fragmentation (Figure 6H) and TUNEL positive cells (Figure 6I). Moreover, conditioned medium (CM) obtained upon induction of c-Kit-EC-stable HCT116 cell line was able to induce an increase of Annexin V-positive population (Figure S4C) and DNA fragmentation (Figure S4D) in parental HCT116 cells, compared to control CM.

To demonstrate that cell death triggered by interfering with SCF/c-Kit binding was due to the pro-apoptotic activity of c-Kit rather than an indirect effect of switching-off the survival kinase-dependent signal, we first used a c-Kit RNA interference strategy. If c-Kit was acting only *via* its kinase activity, silencing of c-Kit should be mimicking the effect of the induction of c-Kit-EC. However, if c-Kit was really acting as a dependence receptor, the silencing of c-Kit should somehow revert the effect of c-Kit-EC. As shown in Figure 7A and S4E, transfection of stable HCT116 cells inducible for c-Kit-EC with c-Kit specific siRNA is sufficient to revert the increase of Annexin V-positive population observed upon induction of c-Kit-EC. To confirm the pivotal role of endogenous c-Kit receptor to induce apoptosis, we generated, using CRISPR/Cas9 strategy, two stable HCT116 cell line knocked-out for c-Kit, obtained by two different c-Kit-specific guides RNA (Figure S4F). While parental HCT116 cell line showed an increase of Annexin V-positive population (Figure 7B) or DNA fragmentation rate (Figure 7C)

following treatment with CM obtained upon c-Kit-EC induction, treatment of c-Kit knocked-out cell lines had no effect on apoptosis induction (Figure 7BC). These results confirmed that cell death induction by interfering with SCF/c-Kit binding was triggered by c-Kit pro-apoptotic activity.

This *in vitro* data supports the view that interfering with SCF/c-Kit interaction could be of interest in therapeutic setting. Stable HCT116 cell line inducible for c-Kit-EC was thus engrafted in SCID mice and animals with tumors were treated daily by i.p. injections of doxycycline, in order to induce c-Kit-EC expression (Figure S4G) and interfere with SCF/c-Kit binding as a mean to activate pro-apoptotic activity of endogenous c-Kit. Doxycycline treatment significantly slowed down tumor growth (Figure 7D) and decreased final tumor sizes (Figure 7EF), compared to vehicle-treated animals. Taken together, these data support the hypothesis that targeting SCF/c-Kit interaction to stimulate c-Kit pro-apoptotic activity could be investigated further as a complementary therapeutic strategy to c-Kit kinase inhibitors.

## Discussion

The proto-oncogene c-Kit has been classically considered only as a receptor tyrosine kinase and most of the studies on c-Kit focused on its kinase activity. Hence, therapeutic strategies targeting c-Kit have been centered on the inhibition of its kinase activity, as exemplified by the development of imatinib (Gleevec), blocking ATP binding to c-Kit active site (Buchdunger et al., 2002). Although imatinib has been proved to be effective in several cancer such as c-Kit positive Gastrointestinal Stroma Tumors (GIST), 10% of patients show primary resistance and a vast majority of patients eventually develop resistance to the drug, due to mutations in KIT that render GIST cells imatinib-resistant and allow c-Kit signaling to remain constitutively active (Corless et al., 2011). We propose here that rather than inhibiting c-Kit kinase activity, an alternative therapeutic strategy could be to target the interaction between SCF and c-Kit to both inhibit the kinase activity and to induce c-Kit-induced cell death, at least in patients showing an autocrine/paracrine production of c-Kit ligand.

Several hints have led us to formulate this hypothesis. Indeed, it has been shown that removal of SCF or addition of a monoclonal c-Kit antibody trigger apoptosis in murine melanocyte precursors (Ito et al., 1999). Moreover, SCF promotes mast cell survival by suppressing apoptosis (Iemura et al., 1994). In addition, c-Kit expression is lost in neuroblastoma (Krams et al., 2004), a disease that originates from migrating neural crest cell, from which also melanocyte precursors arise. Along this line, the loss of expression of c-Kit receptor may be related to neuroblastoma disease progression (Shimada et al., 2008). Furthermore, c-Kit down-regulation has been described in invasive melanoma, thyroid

carcinoma and breast cancer (Natali et al., 1995; Natali et al., 1992a; Natali et al., 1992b). These findings did not fit easily with a typical profile of a proto-oncogene. This matches much better with c-Kit being a dependence receptor. The pro-death activity of dependence receptors has been shown to confer a tumor suppressor activity to these receptors and accordingly they are often lost or down-regulated in several human cancers, including colorectal, breast, ovary, uterus, stomach, lung or kidney cancers (Bernet et al., 2007; Genevois et al., 2013; Mehlen and Fearon, 2004; Thiebault et al., 2003). Of interest while in a large fraction of cancer, dependence receptors are down-regulating, in other cancers, a similar selective advantage is the gain of the ligand (Bouzas-Rodriguez et al., 2010; Delloye-Bourgeois et al., 2009; Delloye-Bourgeois et al., 2013; Fitamant et al., 2008; Paradisi et al., 2013; Paradisi et al., 2008). In this setting, the autocrine production of the ligand of a dependence receptor represents a survival advantage inhibiting tumor cell death and accordingly promoting tumor progression and metastasis (Mehlen et al., 2011). Targeting ligand/dependence receptor interaction in cancer with up-regulation of DR ligand is currently assessed in early clinical trial for the pair netrin-1/UNC5B (<https://clinicaltrials.gov/ct2/show/NCT02977195>). Of interest, while c-Kit amplification and constitutive kinase activation is one of the major modes of action for promoting tumor progression, an autocrine production of SCF has also been extensively described (Lennartsson and Ronnstrand, 2012; Theou-Anton et al., 2006). Even though so far this gain of SCF is mainly seen as a mechanism to promote c-Kit kinase activation, our data suggest that may also block c-Kit-induced apoptosis.

Including c-Kit in the functional family of dependence receptors may have important therapeutic consequences. Indeed, in cancers showing SCF expression, inhibiting the kinase

activity may not be sufficient to efficiently trigger cell death of tumor cells. Thus, a co-treatment based on both kinase inhibition and stimulation of the pro-apoptotic activity of these tyrosine kinase dependence receptors could appear as a more attractive and efficient therapeutic strategy that may bypass some of the currently observed tumor resistance. We have started here to validate this hypothesis, using c-Kit extracellular domain to induce cell death and tumor growth inhibition in SCF-expressing HCT116 cancer cells. Of interest, along our hypothesis is the observation that the c-Kit caspase cleavage mutation appears to be detected in human tumor upon resistance to Gleevec. Even though so far the mechanism of action for this mutation is generally considered as the change in accessibility of Gleevec to c-Kit (Gajiwala et al., 2009), further studies should investigate whether D816V mutation is also selected because it prevents c-Kit to eliminate cancer cells. Further work will unravel whether combining a kinase inhibitor and a SCF/c-Kit blocking biologic could be actually more efficacious and associated with less resistance.

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## **Authors Contribution**

HW has performed and analyzed most of the experiments. AB, LM, STD, YS, DG have contributed to some important experiments. AP and PM have supervised the work and have written the manuscript.

## **Declaration of Interests**

The authors declare to have no conflicts of interest related to this work.

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## Figure Legends:

### Figure 1. c-Kit induces apoptosis *in vitro*.

**(A):** c-Kit over-expression triggers cell death. A549, IMR32 and HMCB stable cell lines inducible for wild-type (wt) or kinase mutated (kinase mut) c-Kit were treated with the indicated amount of doxycycline (Dox) in 96-well-plate (A549 and IMR32) or 6-well-plate (HMCB). Cell death was analyzed by propidium iodide (PI) staining 36 hours (IMR32 and HMCB) or 48 hours (A549) after doxycycline treatment, using Incucyte Zoom instrument. Expression of c-Kit proteins upon doxycycline treatment was confirmed by western blot (bottom panel). **(B):** c-Kit-induced cell death is associated with phosphatidylserine flipping to the extracellular surface of the cell, a specific hallmark of apoptotic cell death (Vermes et al., 1995). IMR32 and HMCB stable cell lines were treated with doxycycline and apoptosis rate was quantified by AnnexinV/PI staining through flow cytometry. Top right quadrant represents double positive cells (late apoptosis), while cells positive only for Annexin V (early apoptosis) are present in bottom right quadrant. Number in the quadrants correspond to percentage of cells. **(C,D):** c-Kit is able to activate caspase-3. IMR32 and HMCB stable cell lines were treated with doxycycline to induce c-Kit and active caspase-3 was quantified by fluorometric detection kit (C). Caspase-3 activity was normalized to total protein amount and indexed to untreated cells. Caspase-3 activation was also evaluated by western blot (D), using a caspase-3 antibody able to recognize uncleaved (inactive) or cleaved (active) protein. **(E):** c-Kit promotes Mitochondrial Outer Membrane Permeabilization (MOMP). HMCB stable cell line was treated

with doxycycline and mitochondrial depolarization rate was quantified by JC-1 dye staining. Low right quadrant represents mitochondrial depolarized cells. Number in the quadrants corresponds to percentage of total cells. Data are representative of at least three replicates as mean  $\pm$  SEM. ns, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . wt, wild-type; Dox, doxycycline; PI, Propidium Iodide.

**Figure 2. c-Kit induces caspase-dependent apoptosis.**

**(A,B):** c-Kit-induced apoptosis requires caspase-3 activation. HMCB (A), A549 and IMR32 (B) stable cell lines were treated with doxycycline and the pan-caspase inhibitor Q-VD-OPh hydrate (Q-VD) or the specific caspase-3 inhibitor, and cell death was evaluated by DNA fragmentation assay (A) or by Propidium Iodide (PI) staining (B). **(C):** Caspase-3 and caspase-9 deletion protects cells from c-Kit-induced apoptosis. Caspase-3 and caspase-9 were knocked-down separately in HMCB stable cell line using CRISPR-Cas9 technique, assessed by three caspase-3 or caspase-9 guide RNAs. The knocking-down efficiency of these guides RNAs were evaluated by western blot (left panels). sgRNA-3 for caspase-3 and sgRNA-2 for caspase-9, showing a better knock-down efficiency, were chosen for further experiments. HMCB stable cell lines were then treated with doxycycline and cell death was evaluated by DNA fragmentation assay (right panel). Data are representative of at least three replicates as mean  $\pm$  SEM. \*\*,  $p < 0.01$ . Dox, doxycycline; PI, Propidium Iodide.

**Figure 3. c-Kit-induced apoptosis is inhibited by SCF and is mediated by intracellular caspase-like cleavage driving a caspase activation amplification loop**

(A,B): The ligand SCF inhibits c-Kit-induced cell death. A549 and IMR32 stable cell lines inducible for kinase-mutated c-Kit were treated with doxycycline and recombinant SCF proteins, derived from *E.coli* (R&D, 1 $\mu$ g/ml) or from 293T cells (cs, 25ng/ml). Apoptotic cell death was then evaluated in A549 stable cell line by propidium iodide (PI) staining 48 hours after treatment, and quantification by Incucyte Zoom instrument (A), and in IMR32 stable cell line by caspase-3 activation, quantified by fluorometric detection kit (B). (C,D,E,F): c-Kit is cleaved by caspase-3. The *in vitro*-translated intracellular region of wild-type (wt) or caspase-cleavage site mutated (D816V) c-Kit were incubated with 0.3 $\mu$ M of purified active caspase-3 and digested bands were analyzed by autoradiography (C). While wt c-Kit cleavage revealed the presence of two major bands at 31kDa (corresponding to c-Kit fragment from transmembrane domain to D816) and 18kDa (c-Kit fragment to D816 to stop codon), these bands disappeared when using D816V mutant. c-Kit cleavage was then confirmed in IMR32 cells transfected with kinase-mutated (D816) or kinase- and caspase-mutated (V816) intracellular region of c-Kit (D). After treatment for 2 hours with the proteasome inhibitor MG132, western blot analysis using a C-terminal HA tag revealed the presence of a band at 18kDa that disappeared in V816 construct. The cleavage at D816 site of full-length c-Kit was also confirmed by using IMR32 stable inducible cell lines (E), in which c-Kit or its mutants was induced by doxycycline. After a 2-hours treatment with the proteasome inhibitor Bortezomib, western blot analysis using an antibody targeting the C-terminal region of c-Kit revealed the presence of a band at 18kDa in wild-type (wt) and kinase-mutated (D792N) c-kit, but not in

the c-Kit stable cells mutated at D816 site. Western blot presented here was representative of three independent experiments. In addition, endogenous c-Kit could also be cleaved (F). In M2Ge melanoma cell line, an 18kDa band was detected by c-Kit antibody targeting the C-terminal region, in presence of Bortezomib. **(G,H)**: c-Kit interacts with and activates caspase-9. 293T cells were transfected with kinase-mutated c-Kit and caspase-9-HA plasmids. After lysis, caspase-9 was pulled-down by HA antibody and c-Kit was detected in the immunoprecipitated fraction using  $\alpha$ -c-Kit antibody (G). IMR32 stable cells, inducible for kinase-mutated c-Kit, were treated with doxycycline for 24 hours and, after lysis, kinase-mutated c-Kit was pulled down by Flag antibody. Active caspase-9 that bound to pulled down c-Kit was quantified by caspase-9 activity detection kit (H). Data are representative of at least three replicates as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; wt, wild-type; Dox, doxycycline; PI, Propidium Iodide; R&D, R&D systems; cs, Cells Signaling; IP, Immunoprecipitation; IC, intracellular region.

**Figure 4. c-Kit-induced apoptotic cell death requires c-Kit caspase-3 cleavage.**

**(A,B,C)**: c-Kit caspase cleavage is required to cell death induction. IMR32 stable cell line inducible for kinase-mutated (mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit were treated with the indicated doxycycline concentrations. Apoptotic cell death was then evaluated by PI staining 36 hours after doxycycline treatment in 96-well plate (A) and by AnnexinV/PI double staining analyzed by flow cytometry (B). In addition, D816V c-Kit was not anymore able to induce Mitochondrial Outer Membrane Permeabilization (MOMP), promoted by kinase-mutated c-Kit, as indicated by mitochondrial depolarization quantified by JC-1 dye staining (C). **(D,E)**: D816V mutation impairs the formation of the caspase-activating complex

between c-Kit and caspase-9. 293T cells were transfected with kinase- or double-mutated c-Kit and caspase-9-HA plasmids (D). After lysis, caspase-9 was pulled-down by HA antibody and c-Kit was detected in the immunoprecipitated fraction using  $\alpha$ -c-Kit antibody. Moreover, IMR32 stable cells, inducible for kinase- or double-mutated c-Kit, were treated with doxycycline for 24 hours and, after lysis, kinase-mutated c-Kit was pulled down by Flag antibody (E). Active caspase-9 was then quantified in the pulled-down fraction by caspase-9 activity detection kit. Data are representative of at least three independent experiments as mean  $\pm$  SEM. *ns*, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Dox, doxycycline; PI, propidium iodide.

**Figure 5. c-Kit over-expression induces tumor regression *in vivo***

**(A,B,C):** SCID (severe combined immunodeficiency) mice engrafted with HMBC kinase mutated (mut) c-Kit stable cell line were intraperitoneal treated everyday with doxycycline (50mg/kg diluted in H<sub>2</sub>O) or vehicle. Tumor sizes were measured at the indicated days post xenograft (A) and after the mice were sacrificed (B). Apoptotic cells in tumor sections revealed by TUNEL staining are indicated by the red arrows (representative images) and TUNEL-positive cells were quantitated (C). **(D,E,F):** HMBC stable cell line inducible for kinase-mutated (mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit was engrafted in SCID mice. Mice were intraperitoneal treated everyday with doxycycline (50mg/kg diluted in H<sub>2</sub>O) or vector. Tumor sizes were measured at the indicated days post xenograft (D) and after the mice were sacrificed (E). Photos of representative mice are shown right before sacrificing the mice (F). Tumors were indicated in the red circles. Data are representative as mean  $\pm$  SEM. *ns*, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.0001$ . Dox, doxycycline.

**Figure 6. SCF silencing or interfering triggers apoptosis**

**(A,B):** SCF silencing induces apoptotic cell death. A549 cells were transfected either with a scramble siRNA (siCTL) or with two specific siRNAs targeting SCF (siSCF-1 and siSCF-2). Apoptosis was evaluated by caspase-3 activity, measured 24 hours after siRNA transfection by fluorometric detection kit (A) and by PI staining quantified with Incucyte Zoom instrument (B). Data are representative of at least three independent experiments. **(C):** Combining SCF silencing and Imatinib treatment triggers apoptosis in resistant cells. M2Ge melanoma cells were transfected either with a scramble siRNA (siCTL) or with a specific siRNA targeting SCF (siSCF). 24 hours after SCF silencing, cells were treated with Imatinib for further 24 hours and apoptosis rate was measured by DNA fragmentation assay through flow cytometry. **(D):** Schematic representation of the mode of action of SCF interference by the extracellular region of c-Kit. The binding between SCF and c-Kit could be inhibited by the extracellular domain of c-Kit (c-Kit-EC), which interacts with SCF, acting as a trap able to interfere with SCF/c-Kit binding and thus it could trigger apoptotic cell death induced by unbound c-Kit receptor. **(E):** c-Kit-EC is secreted in the extracellular *milieu*. HCT116 stable cells inducible for c-Kit-EC were treated with the indicated concentrations of doxycycline and the presence of the recombinant protein was evaluated by western blot in the extracellular medium (top) or in cell lysate (bottom). **(F,G,H,I):** Over-expression of c-Kit-EC triggers apoptotic cell death. HCT116 stable cell line inducible for c-Kit-EC was treated with the indicated doxycycline concentrations. Induction of cell death was then evaluated by PI staining measured by Incucyte Zoom device (F), by AnnexinV/PI double staining by flow cytometry (G), DNA fragmentation (H) and

TUNEL (I) assays. Data are representative of at least three independent experiments as mean  $\pm$  SEM. \*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Dox, doxycycline; EC, extracellular domain.

**Figure 7. SCF interference triggers c-Kit-dependent apoptosis and limits tumor growth *in vivo*.**

**(A):** Silencing c-Kit expression is sufficient to inhibit c-Kit-EC-induced cell death. HCT116 stable cell line inducible for c-Kit-EC was transfected with scramble siRNA (siCtl) or c-Kit-specific siRNA (siKIT). 24 hours after transfection, cells were treated with doxycycline and cell death was evaluated by AnnexinV/PI double staining using flow cytometry. Data are representative of three independent experiments. **(B,C)** Deletion of c-Kit gene protects cells from c-Kit-EC induced apoptosis. c-Kit gene was knocked-down in parental HCT116 cell line using CRISPR-Cas9 technique, assessed by two c-Kit-specific guide RNAs (sgRNA-1 and -2). Transgenic cell lines were then treated with conditional medium obtained from stable HCT116 cells inducible for c-Kit-EC without (control) or with (c-Kit-EC) addition of doxycycline. Cell death was evaluated by AnnexinV/PI double staining (B) and DNA fragmentation (C) by flow cytometry. **(D,E,F):** HCT116 stable cell line inducible for c-Kit-EC was engrafted in SCID mice. Mice were intraperitoneal treated everyday with doxycycline (50mg/kg diluted in H<sub>2</sub>O) or vector. Tumor sizes were measured at the indicated days post xenograft (D) and after the mice were sacrificed (E). Photos of representative mice were taken right before sacrificing the mice (F). Tumors were indicated in the red circles. Data are representative of at least three independent experiments as mean  $\pm$  SEM. *ns*, non-significant; \*,  $p < 0.05$ . Dox, doxycycline; EC, extracellular domain.

### Supplemental Figure 1.

**(A):** Kinase-mutated c-Kit cannot be activated by SCF. A549 stable cells inducible for wild-type (wt) or kinase-mutated (kinase mut) c-Kit were treated with doxycycline in the presence or in the absence of SCF. c-Kit activity was evaluated by its ability to be autophosphorylated in presence of SCF, as well as by phosphorylation of ERK1/2. **(B):** Over-expressed wild-type and mutant c-Kit proteins are expressed in cellular membrane. IMR32 stable cell line inducible for wild-type (wt) kinase-mutated (kinase mut) and kinase- and caspase-mutated (D792N/D816V) c-Kit was treated with doxycycline for 24 hours. Membrane staining of c-Kit proteins was then performed by flow cytometry of non-permeabilized cells. **(C):** c-Kit stable inducible cell lines express endogenous-like protein levels. Wild-type (wt) and kinase-mutated (kinase mut) c-Kit were induced in IMR32 stable inducible cells by adding 0.02 or 0.05 $\mu$ g/ml of doxycycline, and protein levels were compared to endogenous c-Kit expressed in M2Ge and XPC melanoma cells. Western blot was performed using a c-Kit antibody targeting the C-terminal region of the receptor. **(D,E):** Ectopic c-Kit expression induced by low doxycycline concentration is sufficient to trigger apoptosis. Stable IMR32 cells were treated with the indicated Doxycycline concentrations for 6 hours and caspase activity was evaluated by fluorometric detection kit (D). Cell death induction by low-expressed ectopic c-Kit was confirmed by PI staining measured by Incucyte Zoom device (E). **(F):** c-Kit-induced cell death requires active caspase-3. HMCB stable cell line inducible for kinase-mutated c-Kit were treated with doxycycline in presence of increasing

concentrations of the pan-caspases inhibitor Q-VD-OPh (Q-VD). Apoptotic rate was evaluated by AnnexinV/PI double staining by flow cytometry. **(G,H):** Deletion of caspase-3 and caspase-9 protects cells from c-Kit-induced apoptosis. Caspase-3 and caspase-9 were knocked-down in IMR32 stable cells inducible for kinase-mutated c-Kit, using CRISPR-Cas9 technique. The knocking-down efficiency was evaluated by western blot (G). Cell death induction upon doxycycline treatment in control and caspase knocked-down cell lines was evaluated by AnnexinV/PI double staining using flow cytometry (H). In the left panel, a representative experiment is presented, while in the right panel the combined result of three independent experiments is shown. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; Dox, doxycycline; sgRNA, small guide RNA.

### **Supplemental Figure 2.**

**(A,B):** The ligand SCF inhibits c-Kit-induced cell death. IMR32 stable cell lines inducible for kinase-mutated c-Kit were treated with doxycycline and increasing concentrations of recombinant SCF protein (supplied by R&D systems). Apoptotic cell death was then evaluated by caspase-3 activation, assessed by caspase-3 cleavage revealed by western blot (A) and by AnnexinV/PI double staining (B), analyzed by flow cytometry **(C):** c-Kit is cleaved at D816 residue site. Stable A549 cells expressing HA-tagged wild-type (D816) or caspase-mutated (V816) intracellular (IC) c-Kit fragment were treated with the proteasome inhibitor MG132 and the generation of c-Kit cleavage band was evaluated by western blot. **(D):** cleavage of endogenous c-Kit. M2Ge

melanoma cells were treated for two hours with MG132, and the generation of 18kDa-fragment was evaluated by western blot, using a c-Kit antibody targeting C-terminal region. **(E)**: caspase activation promotes c-Kit cleavage. M2Ge cells were treated for six hours with increasing concentrations of caspase activators BV-6 and PAC1. Four hours after, cells were treated with the proteasome inhibitor Bortezomib (BTZ) for further two hours. c-Kit cleaved fragment was then revealed by western blot. **(F,G)**: c-Kit caspase cleavage is required for cell death induction. A549 and IMR32 stable cell lines inducible for kinase-mutated (mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit were treated with the indicated doxycycline concentrations for 48 hours. Apoptotic cell death was then evaluated by both PI staining in A549 cells, using Incucyte Zoom instrument (E) and caspase-3 cleavage by western blot in IMR32 cells (F). *ns*, non-significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Dox, doxycycline; BTZ, Bortezomib; PI, propidium iodide; IC, intracellular region.

### **Supplemental Figure 3.**

**(A,B,C)**: Exogenous c-Kit is induced in engrafted cells upon doxycycline treatment. SCID mice were engrafted with HMxCB stable cells inducible for kinase-mutated (kinase mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit. To study c-Kit stability after induction with doxycycline, mice were sacrificed 24 hours or 48 hours after intraperitoneal injection with 50mg/kg of doxycycline and c-Kit expression was evaluated by western blot on engrafted tumors (A). Then, kinase-mut (B,C) or

D792N/D816V (C) c-Kit protein was assessed by western blot on engrafted tumors obtained from SCID mice treated every day with 50 mg/kg of doxycycline for 10 days. Engrafted tumors were grinded and sonicated in RIPA buffer. Dox, doxycycline; CT, control.

#### **Supplemental Figure 4.**

**(A):** SCF protein expression in siRNA-transfected A549 cell line. Cells were transfected with scramble (sictl) or two SCF-specific (siSCF-1 and siSCF-2) siRNAs. The efficiency of gene silencing was evaluated by western blot. Lupus Ku autoantigen protein p80 (Ku80) was used as housekeeping protein. **(B):** Silencing SCF induces caspase-3 activation in HCT116 cell line. Cells were transfected with scramble (sictl) or SCF-specific (siSCF-1 and siSCF-2) siRNAs. The efficiency of SCF silencing was assessed by western blot (left) 24 hours and 48 hours after transfection. Caspase-3 activation (right) was measured 24 hours after transfection using the fluorometric detection kit. **(C,D):** Conditional medium containing c-Kit-EC induces apoptosis in parental HCT116 cell line. HCT116 stable cell line inducible for c-Kit-EC was treated with doxycycline (c-Kit-EC) or water (control). 24 hours later, conditioned medium (CM) was diluted in normal medium at the indicated concentrations and used to treat parental HCT116 cells. Apoptosis was then evaluated by AnnexinV/PI double staining (C) or DNA fragmentation (D) by flow cytometry. **(E):** KIT gene expression in siRNA-transfected HCT116 stable cell line. Cells were transfected with scramble (sictl) or KIT-specific

siRNA (siKIT). The efficiency of gene silencing was evaluated by quantitative PCR, normalized with TBP gene expression. **(F)**: c-Kit was knocked-down in XPC melanoma cell line by CRISPR-Cas9 technique, using two c-Kit specific guide RNAs. The knocking-down efficiency was evaluated by western blot. **(G)**: SCID mice were engrafted with HCT116 stable cells inducible for c-Kit-EC and treated daily with 50 mg/kg of doxycycline. After 10 days, mice were sacrificed and total RNA and protein were extracted from engrafted tumours. c-Kit-EC mRNA following doxycycline treatment, normalized with TBP gene expression, was then measured by quantitative PCR (left), while c-Kit-EC protein levels were assessed by western blot (right). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.0001$ . Dox, doxycycline; EC, extracellular domain; CM, conditional medium; TBP, TATA Binding Protein.

Figure 1

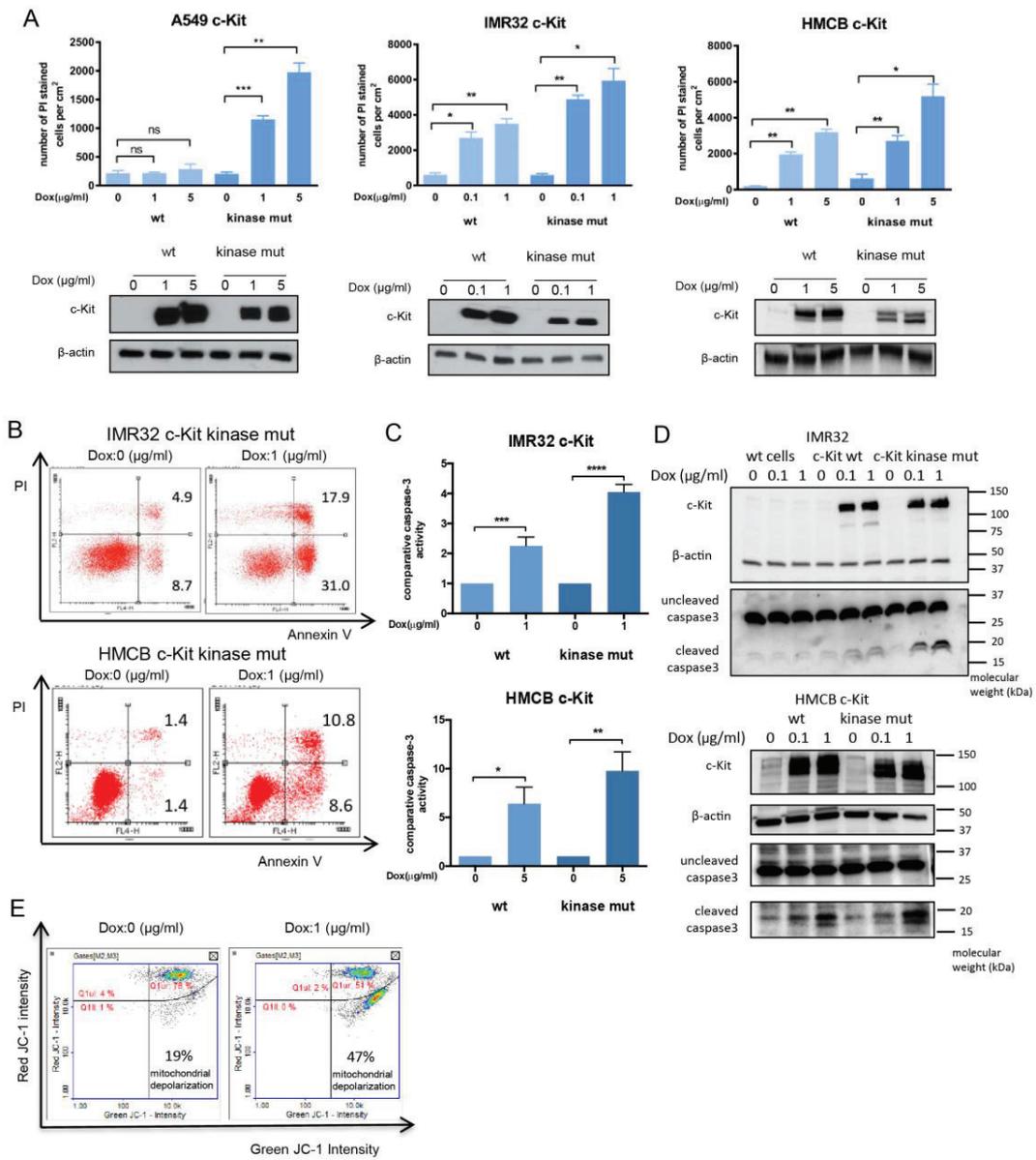


Figure 2

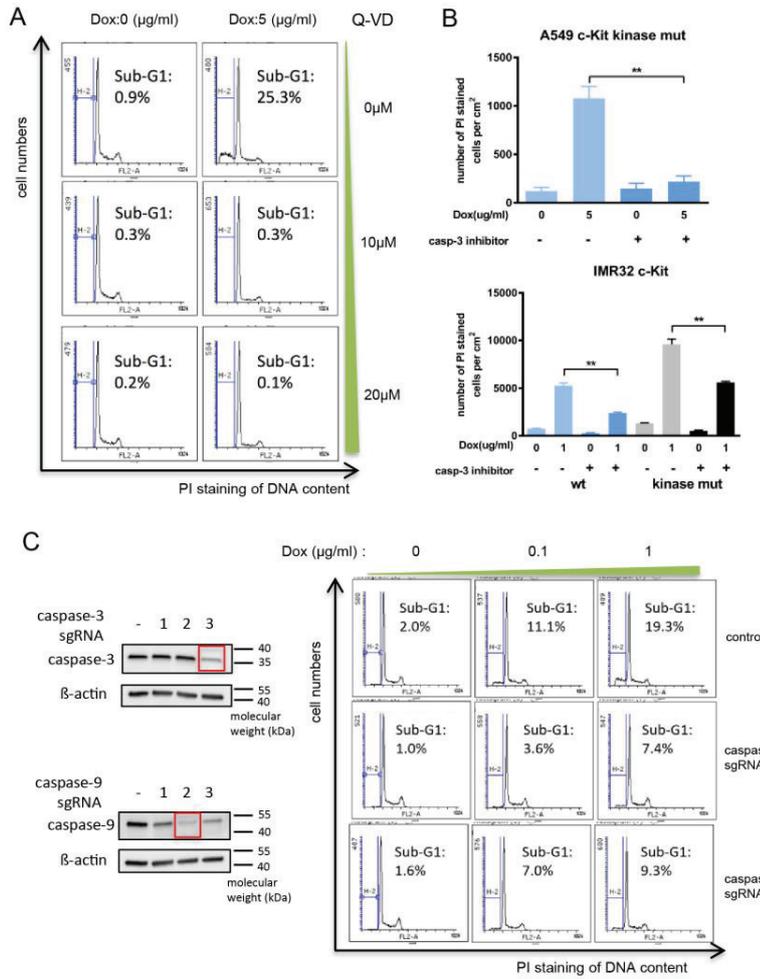


Figure 3

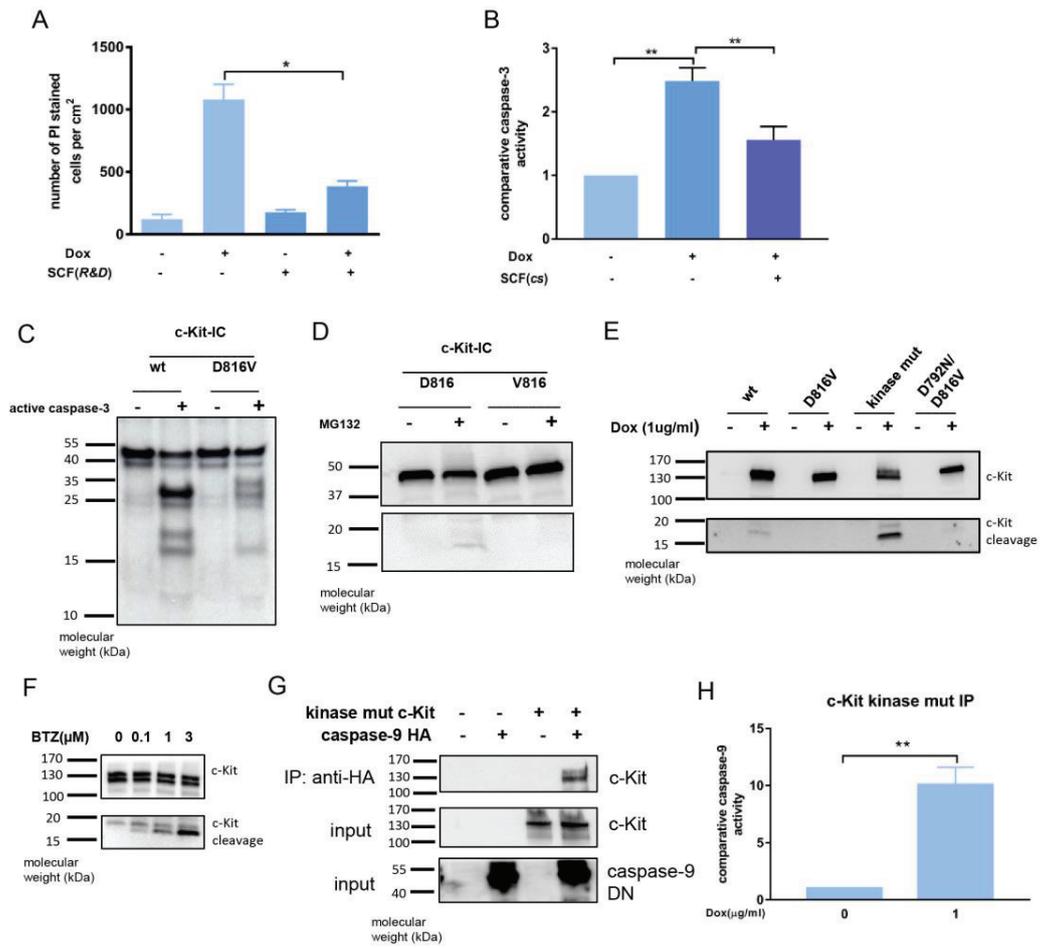


Figure 4

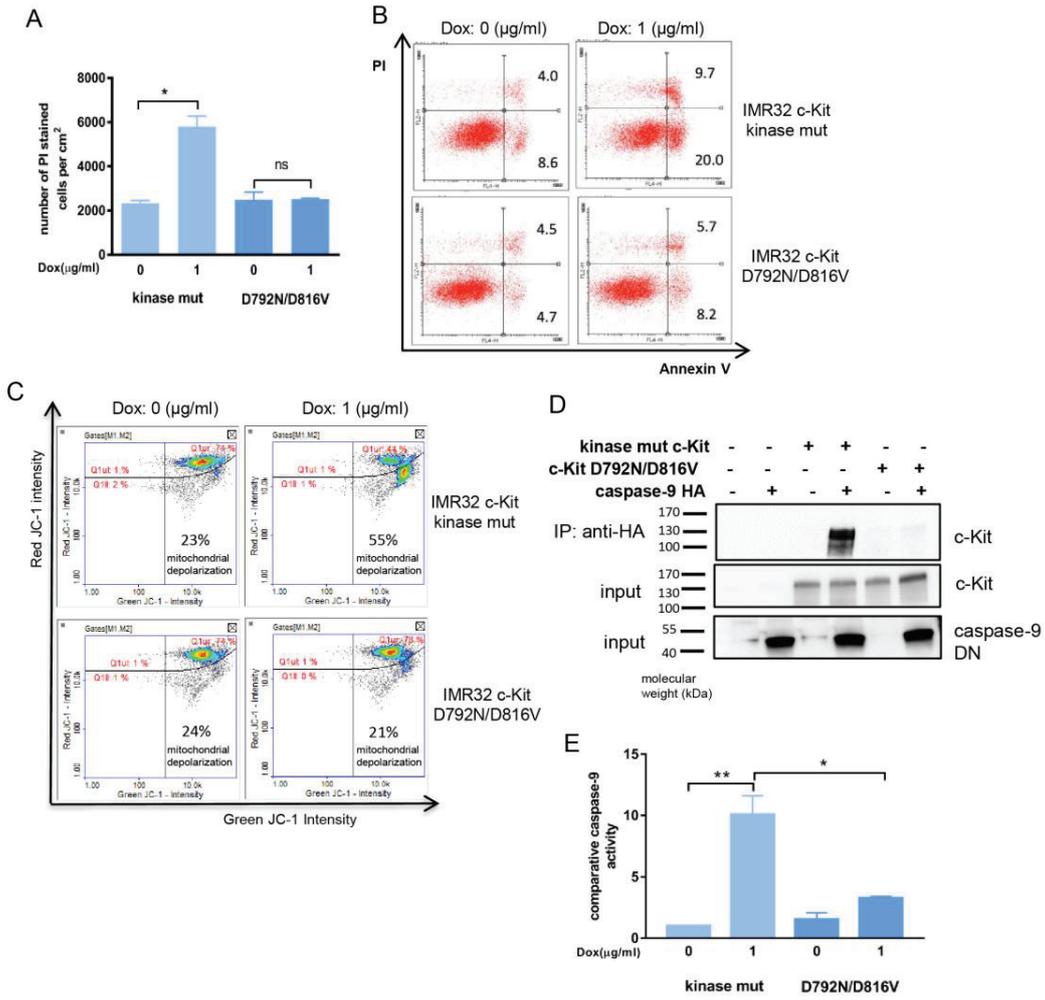


Figure 5

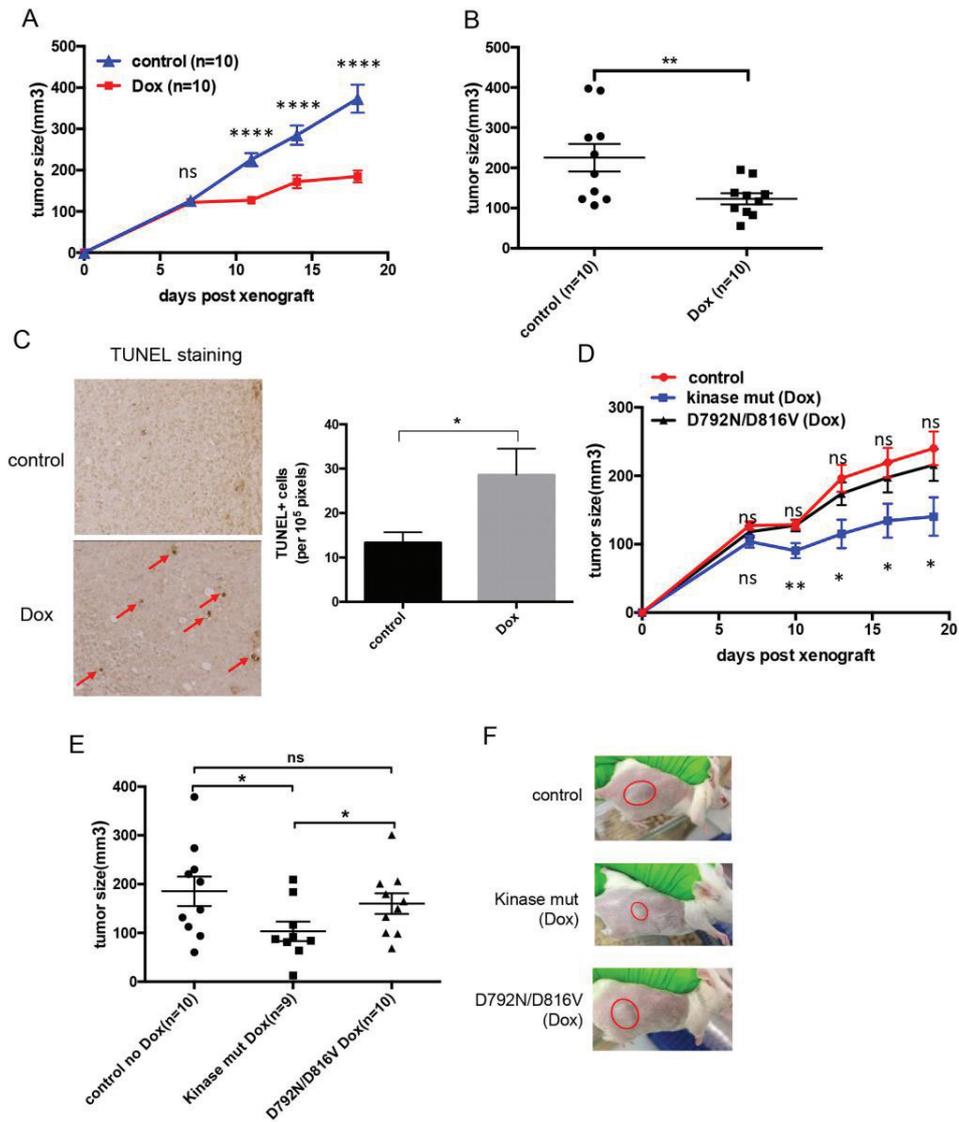


Figure 6

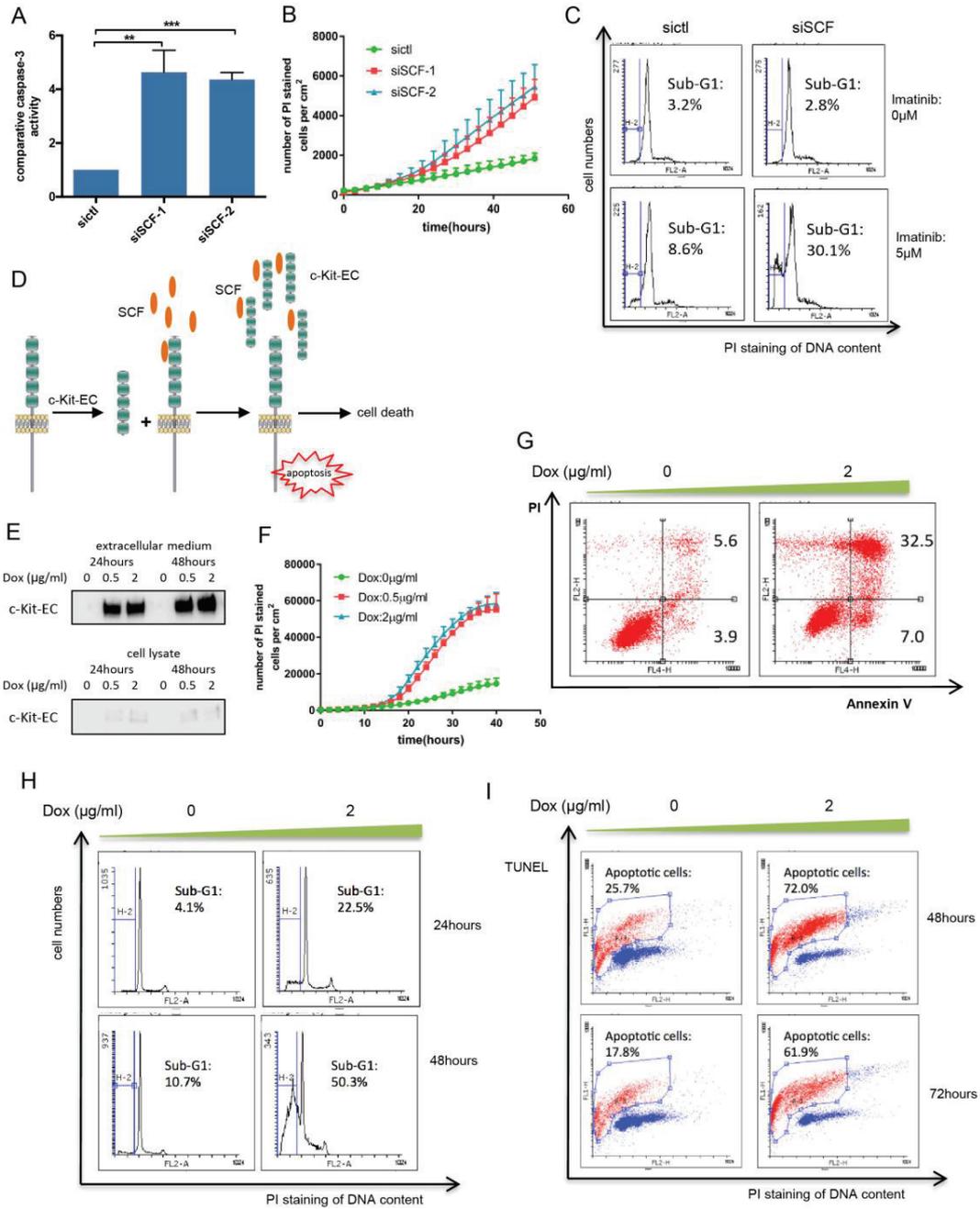
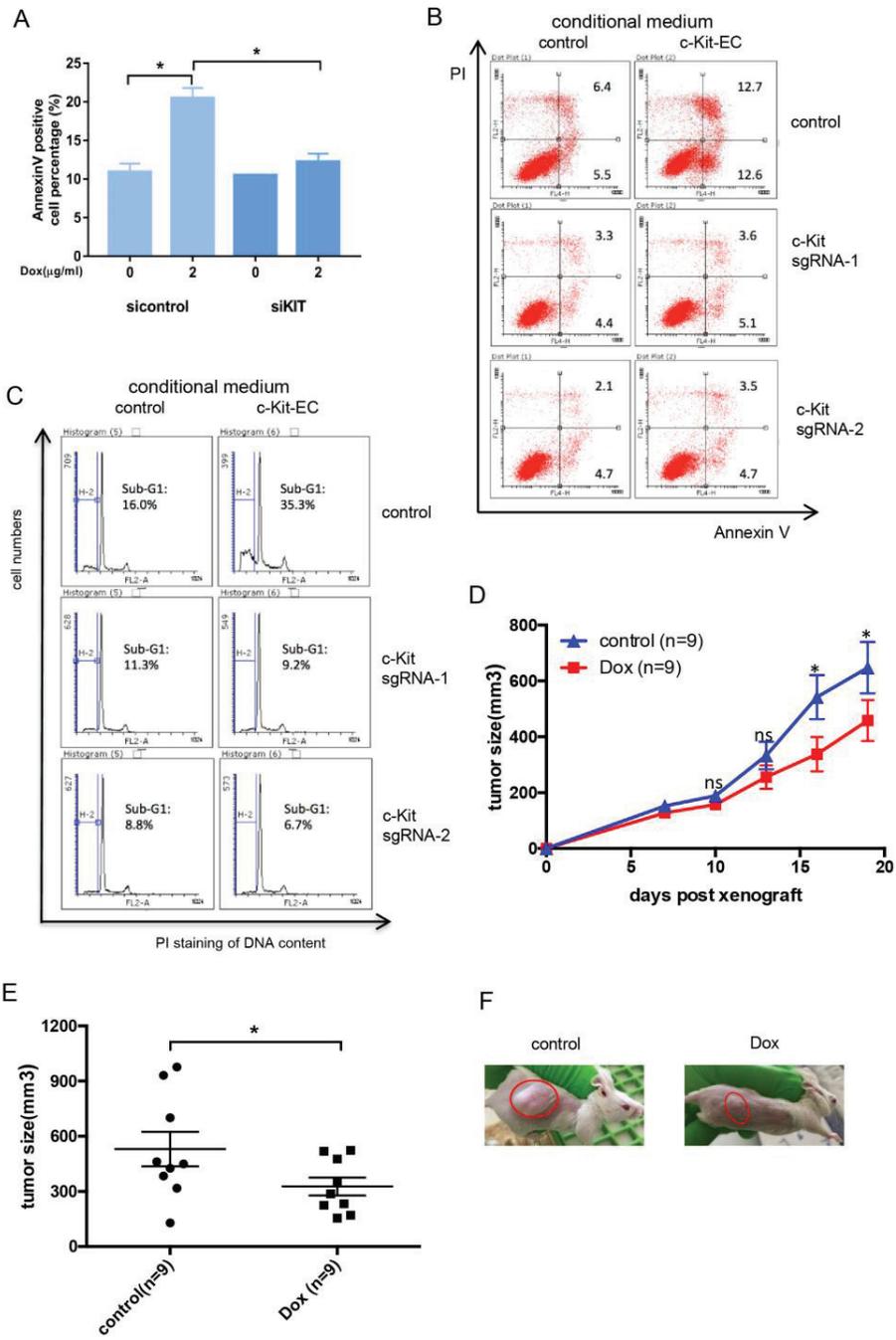
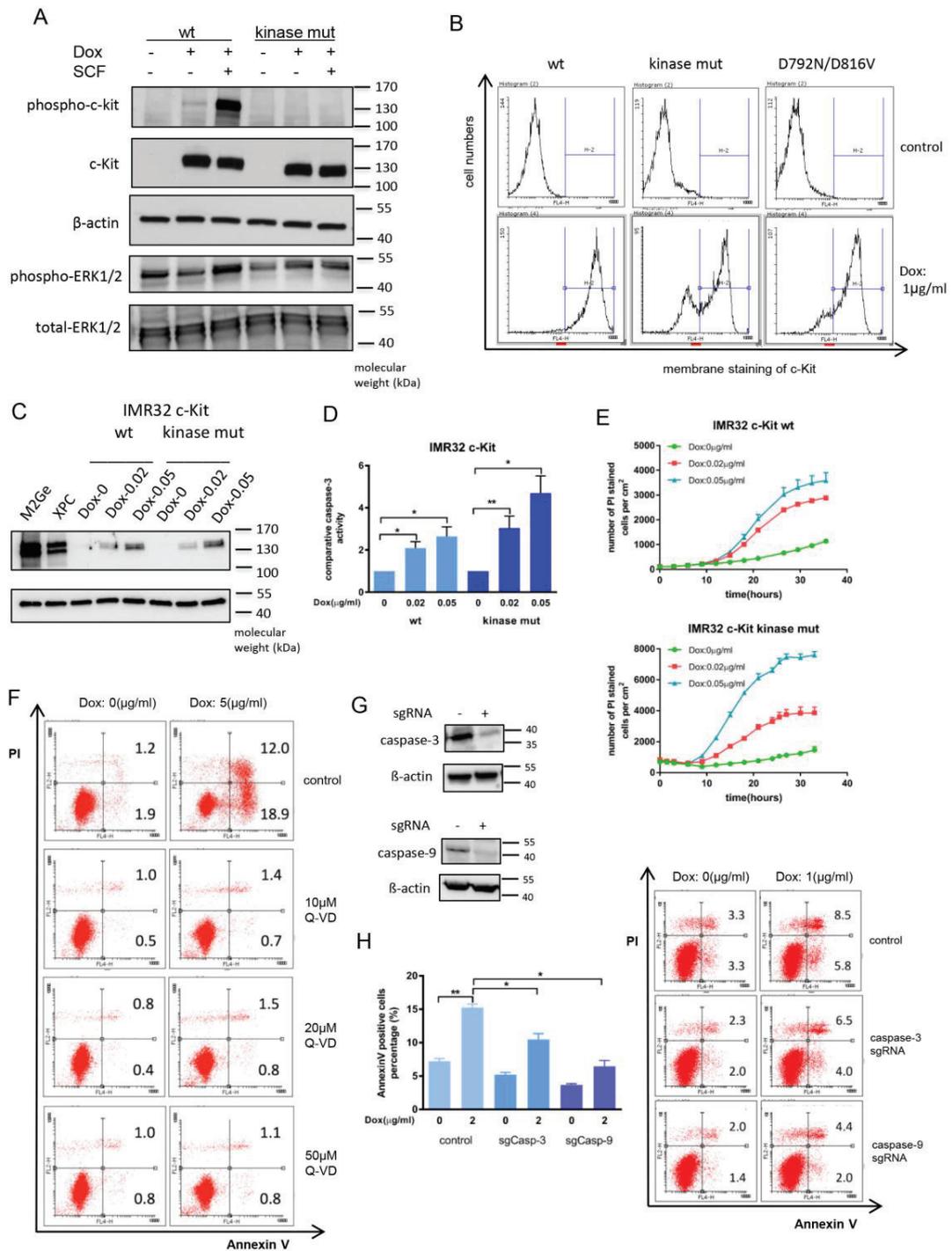
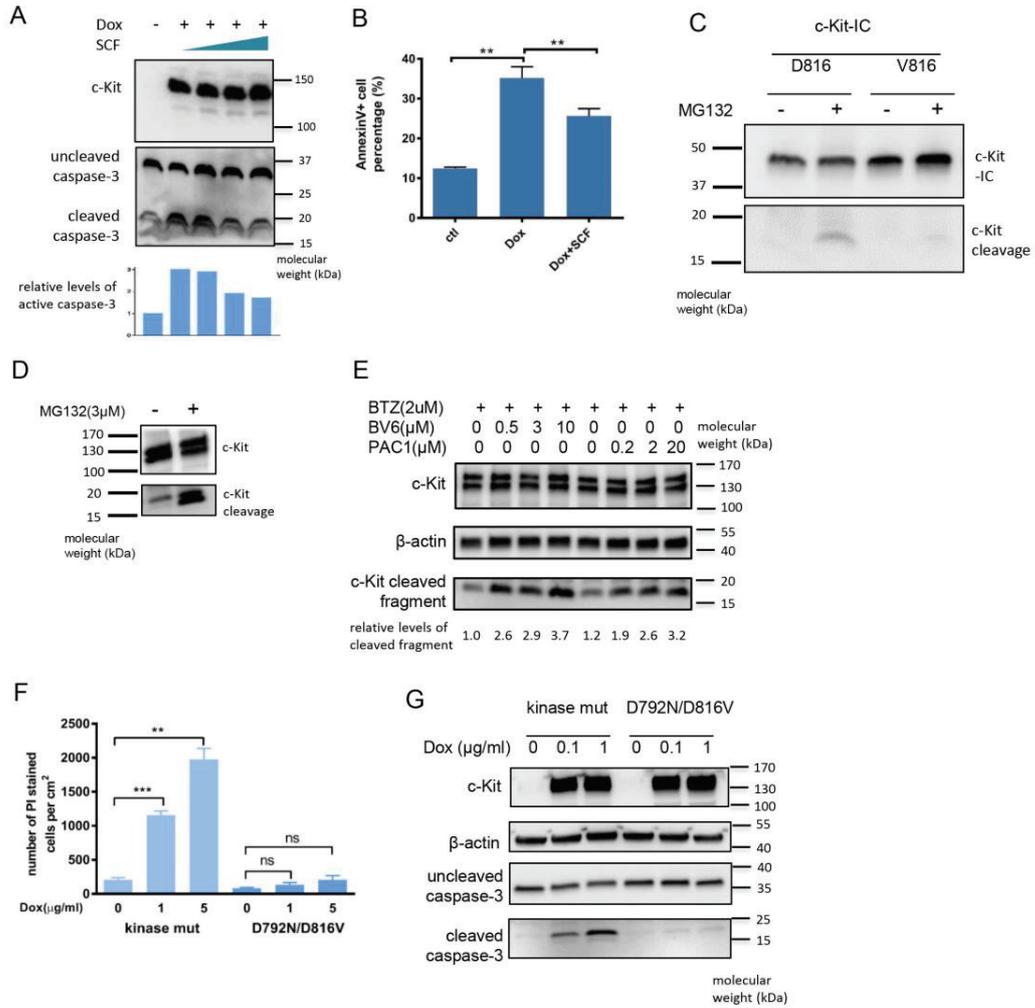


Figure 7

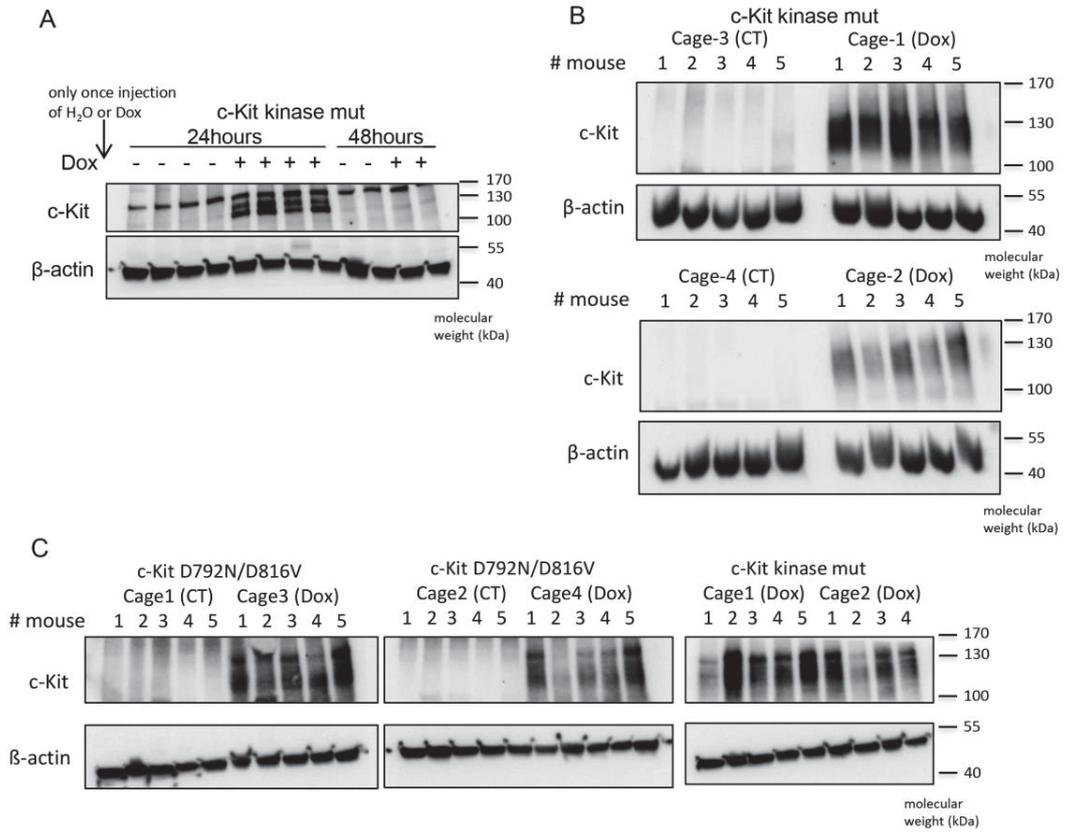




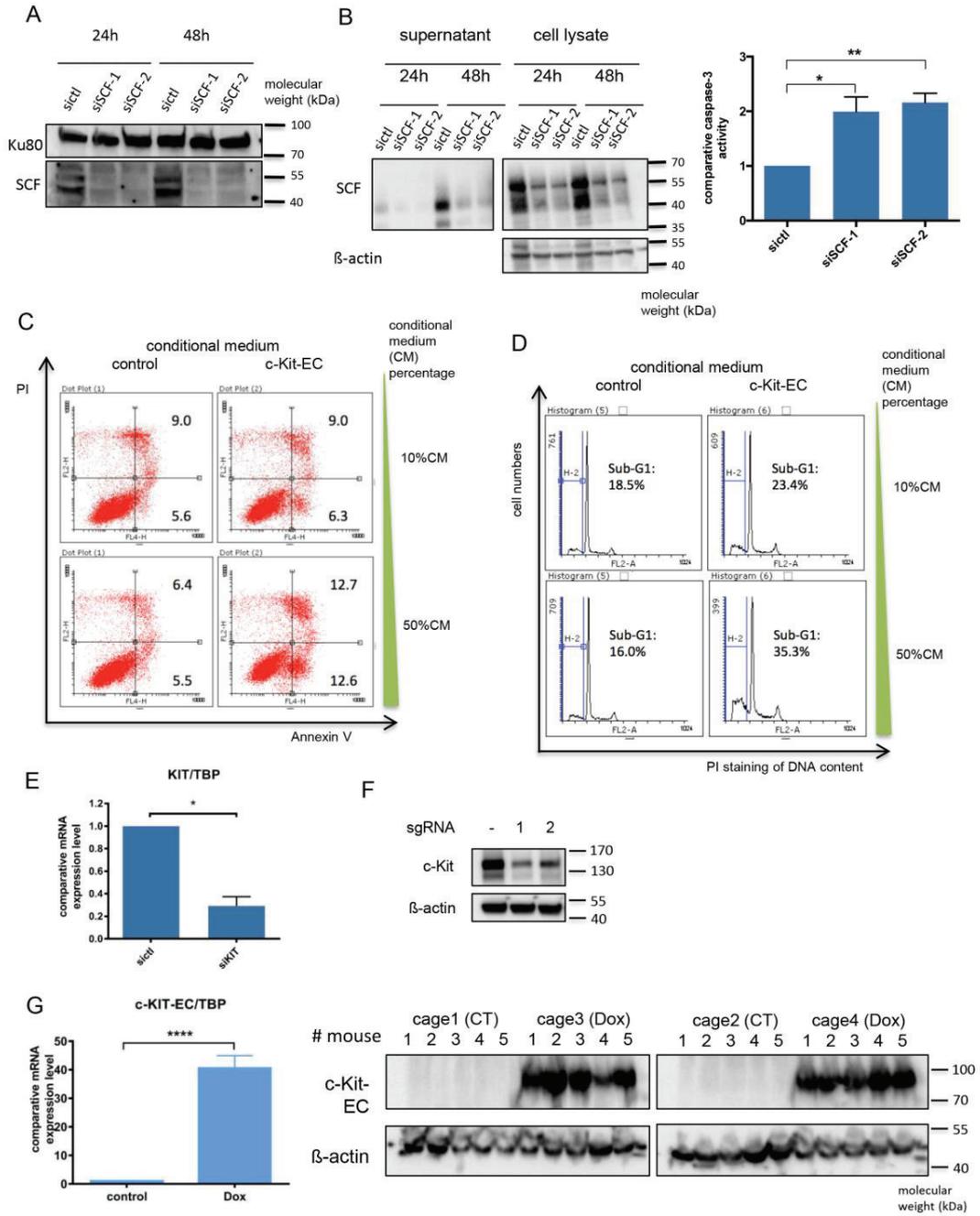
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



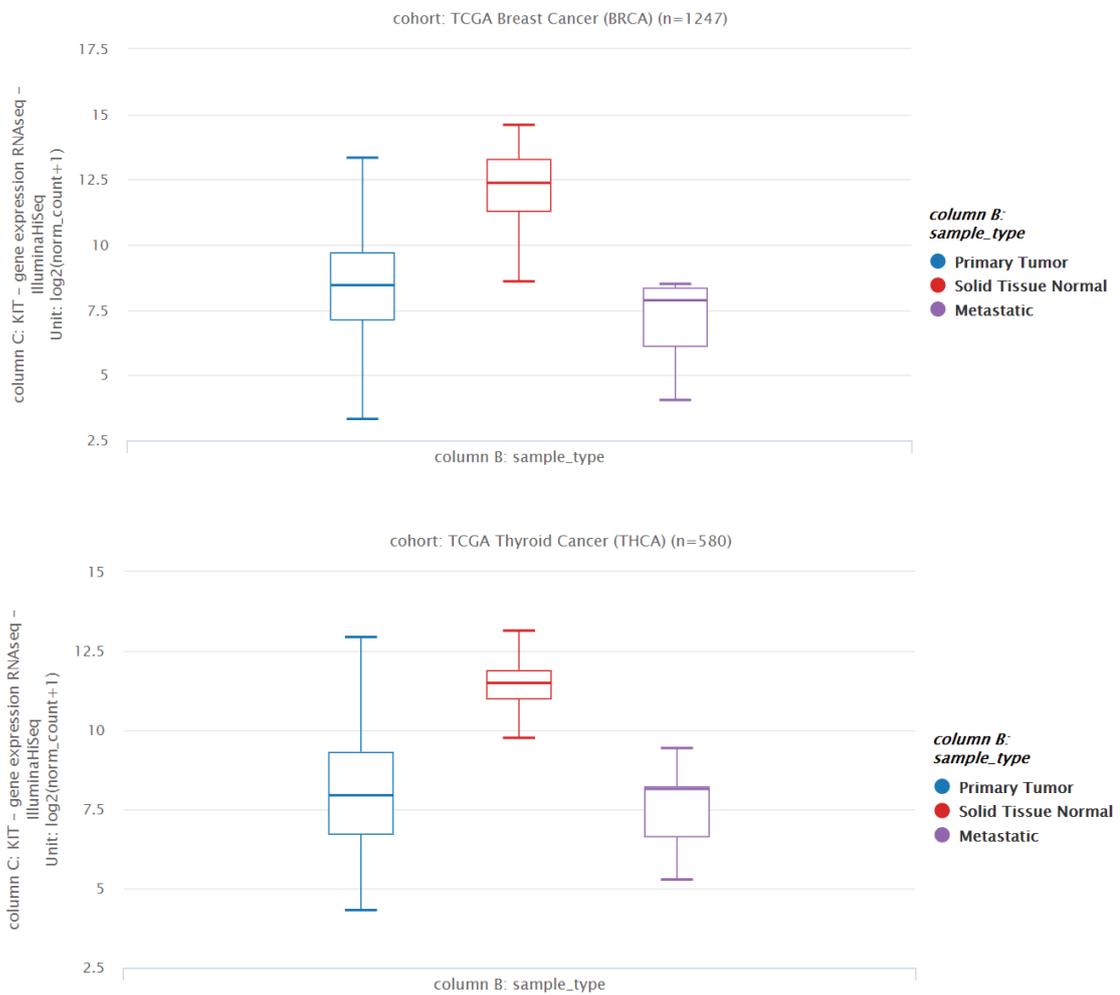
# IV. Discussion and Perspectives

c-Kit has been widely studied as a receptor tyrosine kinase and a proto-oncogene, whose upregulation and mutations lead to tumor progression. However, our results indicate that beside this tyrosine kinase functions, c-Kit is also a member of dependence receptors (DR), whose pro-apoptotic activity strongly depends on their ligands. We demonstrate here that c-Kit is able to actively induce apoptosis when the ligand SCF is absent. The pro-apoptotic activity of c-Kit also relies on its caspase cleavage site D816, the mutation of which can totally silence the pro-apoptotic activity. We also prove that c-Kit is able to bind and activate caspase-9, triggering further apoptotic signaling pathway. Moreover, by adding ligand SCF, cell death induced by c-Kit can be inhibited. Except for these evidences that c-Kit triggers apoptosis *in vitro*, animal models point out that c-Kit also has tumor suppressive effect *in vivo*. At last, we proposed to use the pro-apoptotic activity of c-Kit through blocking SCF/c-Kit binding, in combination with kinase inhibitors, as a novel therapeutic strategy.

### **6.1 c-Kit is downregulated in some types of cancer**

As a classic receptor tyrosine kinase (RTK), c-Kit has been found to be related to numerous kinds of cancers. As described above, upregulation of c-Kit is the cause of several diseases, such as mastocytosis or acute myeloid leukemia (AML). However, the evidences showing a loss of c-Kit expression in some cancers, such as breast cancer (Natali et al, 1992a; Matsuda et al, 1993; Hines et al, 1995; Chui et al, 1996; Palmu et al, 2002; Tsuura et al, 2002; Ko et al, 2003; Simon et al, 2004; Ulivi et al, 2004; Yared et al, 2004; Tsutsui et al, 2006; Tramm et al, 2016) and melanoma (Montone et al., 1997; Shen et al., 2003; Zhu and Fitzpatrick, 2006), supporting our hypothesis indicating c-Kit as a new member of the growing dependence receptor family.

Besides those literature reports, c-Kit has also been found downregulated in some types of cancers in databases. For example, in the TCGA database of breast and thyroid cancer, c-Kit expression level is lower comparing to normal solid tissue (Figure 6.1).



**Figure 6.1** c-Kit expression is downregulated in breast and thyroid cancer. Data from TCGA database. Analysis was performed on platform UCSC Xena.

It is true that c-Kit plays a role as receptor tyrosine kinase in numerous types of cancers and its expression and mutation which deregulate its kinase activity frequently cause cancer progression. However, those evidences, both in literature and in database, made us wonder whether there is a different role of c-Kit in cancer. Combining with the facts that some other RTKs (such as MET, RET and TrkC) are dependence receptors, whose expression is often downregulated in cancers, we came up with the idea that c-Kit could possibly be a candidate for dependence receptor family, which led us to start our project.

## 6.2 Hints from other dependence receptors (DRs) family members

If we propose that c-Kit could be a member of DR family member, it possibly shares the common features of DRs. Thus, we can use the hints from other DR family members to reason the possible characteristics of c-Kit.

Concerning the structure of DRs, actually, there is no common structure domain for all DRs, as DR family is a family of receptors sharing similar functions, not structures. There is only one common sequence that all DRs share - caspase cleavage sequence. Since most DRs contain one or more caspase cleavage sites which are crucial for their pro-apoptotic function, we proposed that c-Kit also contains caspase cleavage site, which is the D816 site (first amino acid in DEVD caspase cleavage sequence). Besides, for other DRs, such as DCC or TrkC, the mutation of the caspase cleavage site silences the pro-apoptotic activity, favoring tumor progression. For example, mutation of the caspase cleavage site of DCC is sufficient to induce neoplasia in intestine and increase the tumor incidence in an adenomatous polyposis coli (APC) mutated mouse model (Castets et al., 2011). Thus, we studied whether the mutation of D816 site on c-Kit could prevent cells from apoptosis induced by c-Kit, which might be a novel explanation why D816 mutation on c-Kit leads to poor prognosis and resistance against treatment.

Another common feature for DRs is that the presence of corresponding ligand is able to revert apoptosis caused by DRs. Besides, these ligands as putative biomarker for the prognostics and potential therapeutic targets (Fitamant et al., 2008; Mancino et al., 2011; Luchino et al., 2013). Hence, we have studied whether the apoptosis induced by c-Kit could be inhibited by adding ligand SCF. Further, in c-Kit positive cells, we demonstrated that by blocking SCF/c-Kit binding, we were able to release the pro-apoptotic activity of c-Kit, which was also able to enhance the effect of imatinib when co-treated.

The mechanisms of other DRs' pro-apoptotic activity also led us to consider possible mechanism for c-Kit. As mentioned in the introduction chapter, most DRs contain, in their intracellular part, a domain required for apoptosis induction. This domain is called the ADD (addiction/dependence domain, as mentioned in the introduction chapter), which is required for cell death induction. Caspase cleavage is responsible for unmasking the ADD. ADD is either in the remaining membrane-anchored fragment or cytosolic generated fragment of the receptor after caspase cleavage, which is dependent on the specific DR. For example, DCC membrane-anchored fragment after caspase cleavage recruits and

activates caspase-9, thereby allowing caspase-3 activation. ADD of Patch is able to interact with DRAL/FHL2, serving as a platform for recruiting TUCAN (and/or NALP1) and caspase-9, further activating caspase-9 apoptotic pathway. On the other hand, TrkC is cleaved by caspases at two sites located at Asp-495 and Asp-641, generating a pro-apoptotic “killer fragment” (TrkC KF) (Tauszig-Delamasure et al., 2007), which interacts with Cobra1, promoting mitochondrial dependent apoptosis (Ichim et al., 2013). Following these examples, we found that c-Kit also contains the ADD in the remaining membrane-anchored fragment after caspase cleavage on D816 site. c-Kit membrane-anchored fragment binds and activates caspase-9, which can be a possible pathway for c-Kit apoptotic activity. As to the cytosolic generated fragment after cleavage, we are not sure yet whether c-Kit has an additional ADD in this fragment. Since the other dependence receptors like TrkC, RET and MET (which are also receptor tyrosine kinases just like c-Kit) have their ADDs on their cytosolic fragments after caspase cleavage (Tauszig-Delamasure et al., 2007; Bordeaux et al., 2000; Tulasne et al., 2004), it is of great interest to study further if c-Kit has the same feature.

### **6.3 c-Kit kinase activity mutation**

As we all know, c-Kit is a receptor tyrosine kinase, which promotes cell survival and inhibits apoptosis when activated by SCF. In our project, it is necessary to overexpress c-Kit for studying its possible pro-apoptotic activity. However, if we overexpress wild-type c-Kit, there is a possible activation of kinase activity, which could disturb or even mask the pro-apoptotic activity expected. Besides, c-Kit is known to form auto-dimerization and phosphorylation when it is overexpressed, even without ligand SCF. Thus, if we want to focus on studying the pro-apoptotic activity of c-Kit, it is necessary to avoid the disturb from kinase activity when inducing c-Kit. That is the reason why we used a kinase mutated form of c-Kit harboring D792N mutation, which was reported to be kinase activity mutated (Roskoski, 2005). Inactivation of c-Kit kinase activity was also verified by evaluating c-Kit auto-phosphorylation, as well as ERK1/2 phosphorylation (see Suppl. Fig.1A). This kinase mutated form of c-Kit allowed us to study the proapoptotic activity of c-Kit, which has been proven by the fact that it triggers stronger apoptosis comparing to wild-type c-Kit without changing the kinase activity.

The choice of using a kinase mutated form of c-Kit is more important when we tried to inhibit c-Kit induced apoptosis by adding ligand SCF to prove that ligand SCF can directly inhibit the pro-apoptotic activity of receptor c-Kit. If SCF was added to cells overexpressing wild-type c-Kit, there should have been a massive activation of kinase activity. Even though SCF might inhibit the apoptosis induced by wild-type c-Kit, we could not make the conclusion that SCF was directly inhibiting the pro-apoptotic activity of c-Kit, because the apoptosis inhibition might just be caused by the kinase activation. On the other hand, if SCF was able to revert apoptosis triggered by kinase mutated c-Kit, we could propose that SCF directly inhibits the pro-apoptotic activity of c-Kit, since no kinase activity change should have occurred.

#### **6.4 c-Kit in different cell lines acts differently**

In our project, many different cell lines have been used as cell models to study the function of c-Kit. It happened frequently that the behavior of c-Kit differed a lot among these cell models, when we studied both endogenous and overexpressed c-Kit.

When we overexpressed wild-type c-Kit, no increase of cell death was observed in A549 cell line, while massive cell death could be seen in IMR32 and HMCB cell lines. On the other hand, after the overexpression of kinase mutated c-Kit, all these three cell lines began to die. Several explanations can be found for this phenomenon. Firstly, as mentioned, c-Kit induces apoptosis when SCF is absent. The cell death induced by c-Kit strongly depends on the SCF autocrine or paracrine. SCF can both directly inhibited the pro-apoptotic activity of c-Kit and indirectly inhibit cell death by activation the kinase activity of c-Kit. Indeed, among the three cell lines mentioned, A549 is the cell line with much more SCF expression comparing to the other two. Thus, it is not surprising that overexpression of wild-type c-Kit was not able to kill A549 cells. Even the kinase mutated c-Kit was able to kill A549 cells, the cell death rate was lower comparing to IMR32 and HMCB cells. The fact that kinase mutated c-Kit still killed some A549 cells even when A549 cells expressed SCF was probably because that part of the overexpressed kinase mutated c-Kit receptors were not bound by SCF, and thus they induced cell death. However, even some wild-type c-Kit might not bind to SCF and thus able to induced apoptosis, it was masked by the positive kinase activity induced by SCF. Secondly, the situation of auto-dimerization and phosphorylation of overexpressed wild-type was

different among cell lines. Thus, in some cell lines, the overexpression of wild-type c-Kit leads to kinase activation that inhibits cell death, while the kinase activity caused by the auto-dimerization in other cell lines might be quite different. Thirdly, even we are not yet totally clear of the possible apoptotic partner or downstream apoptotic pathway except for the caspase-9 binding, it is possible that the apoptotic partners or pathways can vary among different cell lines. If in some cell line the apoptotic partner is absent or mutated, this will affect the pro-apoptotic activity of c-Kit. Fourthly, we have proven in our project that c-kit can induce mitochondrial apoptotic pathway, so the general apoptotic machinery is also an important factor to decide whether c-kit can trigger apoptosis. As we all discussed in the “apoptosis and cancer” chapter in introduction, the expression of apoptotic machinery, such as BCL-2 family members and caspases, varies among different cell lines. Thus, this is also a possible factor affecting to reaction to c-Kit overexpression in different cell models.

In the cases of studying the role of endogenous c-Kit, different cell lines also behave differently. When we knocked down SCF or blocked the SCF/c-Kit interaction, some cell lines, such as HCT116 and A549, began to undergo cell death; while other cell lines, such as M2Ge, stay largely unaffected. One of the explanation is that in some cell lines where c-Kit is highly expressed (the number of c-Kit receptors can be even more than the numbers of SCF ligands, thus many c-Kit receptors are not bound by SCF), c-Kit can form auto-dimerization and phosphorylation, thus these c-Kit receptors trigger kinase signaling independently from ligand SCF. Therefore, when SCF is knocked down in these cell models, c-Kit kinase activity is not largely affected and the possible pro-apoptotic activity is still masked, rendering no cell death.

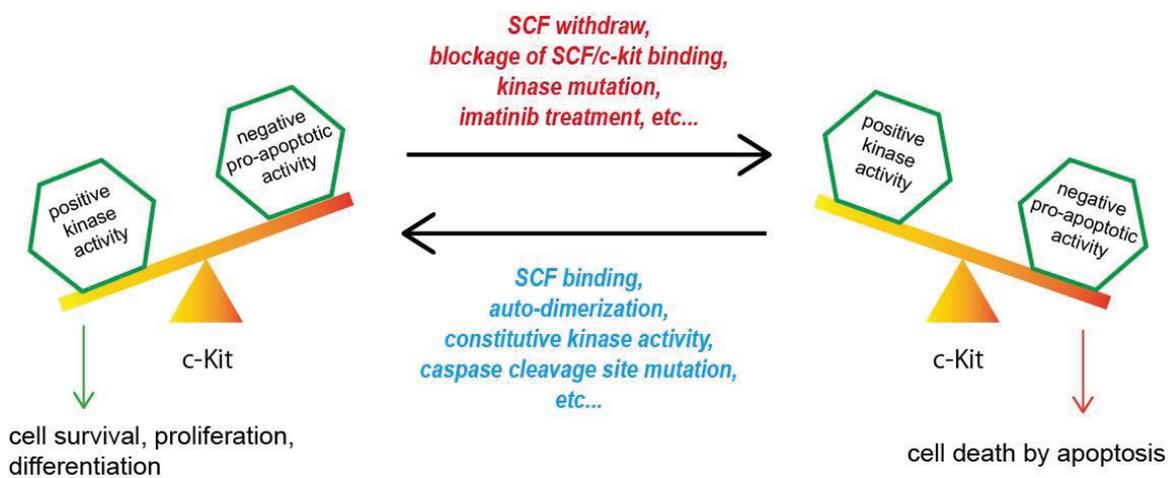
Taken together, c-Kit acts differently among cell lines. Therefore, for each experiment that we performed in our project, it was always a priority to find the appropriate cell models.

## **6.5 c-Kit dual role's balance**

We have shown that c-Kit has a dual role in cells: its “positive” kinase activity promotes cell survival, proliferation, while its “negative” pro-apoptotic activity leads to cell death. There is a balance of this dual role, which is regulated by numerous factors (Figure 6.2).

- 1) On one hand, c-Kit dual role's balance can be switched to apoptotic cell death in various conditions. Firstly, SCF withdraw from c-Kit releases the pro-apoptotic activity of the receptor c-Kit. SCF withdraw can be achieved in several ways. In the experiments performed, we have either knocked down SCF to downregulate SCF, or overexpressed c-Kit so that the majority of c-Kit receptors were not bound by SCF. Both of these two methods created a condition where SCF ligands were absent for most of the receptors. In physiological conditions, SCF withdraw can also happen either by the downregulation of ligand SCF, or by c-Kit positive cells migrating to regions beyond the availability of ligand SCF. Indeed, in the physiological conditions, the cell death caused by SCF withdraw further demonstrates that c-Kit has a tumor suppressive role (Toki et al., 2009; Lee et al., 2000). Secondly, when the SCF/c-Kit binding is blocked in c-Kit expressing cells, endogenous c-Kit can also lead to apoptosis. In our project, we used c-Kit-EC as a trap to block SCF/c-Kit interaction. Theoretically, a blocking antibody which target SCF/c-Kit interaction can also achieve the same effect as c-Kit-EC. Thirdly, kinase activity mutations on c-Kit also frees the pro-apoptotic activity of c-Kit through silencing its positive kinase activity. Indeed, as we have shown in the Results chapter, overexpressed kinase mutated c-Kit usually triggered stronger apoptosis comparing overexpressed wild type c-Kit. Especially in some cells lines (such as A549), overexpressed kinase mutated c-Kit led to cell death, while overexpressed wild-type c-Kit could not. However, the kinase activity mutations are rarely observed in c-Kit related diseases, probably because cells containing these mutations have selective disadvantages or they are even directly eliminated by apoptosis. Fourthly, tyrosine kinase inhibitors (TKIs) treatment (such as imatinib or dasatinib) directly block the c-Kit positive kinase signaling, inducing apoptosis in cells that are dependent on c-Kit positive kinase activity to survive. Some inhibitors (such as imatinib) bind to the ATP binding pocket of c-Kit to directly silence its kinase signaling (Johnson LN, 2009), while other inhibitors (such as dasatinib) target the downstream signaling pathways of RTKs (Shah et al., 2006).
- 2) The factors that favor the balance to cell survival, proliferation and differentiation include but not limit to the following: Firstly, the upregulation of ligand SCF in c-Kit expressing cells directly activates the kinase activity and blocks the pro-apoptotic activity of c-Kit. Indeed, we have demonstrated in the Results chapter that by adding ligand SCF, the apoptosis caused by c-Kit could be prevented.

Physiologically, the upregulation of SCF can also be a factor favoring cell survival and proliferation, thus possibly results in tumor progression. For example, there are reports indicating a significantly increased expression of SCF in hepatocellular carcinoma (HCC), and that SCF is an independent prognostic biomarker for overall and relapse-free survival of patients with HCC (Wang X et al., 2014). Secondly, c-Kit auto-dimerization and auto-phosphorylation independent from SCF could also favor kinase activity without the SCF binding. For example, in some cells where c-Kit expression is high and auto-dimerization can form, even if the ligand SCF is limited or even absent, cells can still survive with the kinase activity offered by auto-dimerization. The auto-dimerization is also a possible reason why in some cell lines (such as A549), the overexpression of wild-type c-Kit rendered no cell death. Thirdly, some mutations on c-Kit juxtamembrane domain and kinase domain can disrupt the autoinhibitory mechanisms of Kit, resulting in constitutively kinase active c-Kit, providing positive kinase activity. (Sakuma et al., 2003; Orfao et al., 2007) Fourthly, as mentioned in previous chapters, caspase cleavage site (D816) mutation on c-Kit is able to prevent pro-apoptotic activity of c-Kit, switching the c-Kit activity balance to cell survival.



**Figure 6.2** The balance between c-Kit positive kinase activity and negative pro-apoptotic activity.

## 6.6 Caspase cleavage site and cleavage band of c-Kit

As a vast majority of dependence receptors, c-Kit also contains a caspase cleavage site, corresponding to Asp in position 816 (D816), which is crucial for its pro-apoptotic activity. By now, we cannot exclude the possibility that c-Kit might have other caspase cleavage sites not yet found. However, D816 mutation is one of the most mutated sites in c-Kit related disease and this mutation is related to poor prognosis in some cancer types. The classic explanation is that D816 mutation leads to c-Kit constitutively active, offering kinase activity without ligand binding. Thus, D816 mutation (especially D816V) leads to pro-tumoral advantage. Another advantage for D816 mutated cells in cancer development is that this mutation might cause resistance against imatinib treatment, rendering a selective advantage for tumor cells. Indeed, in some types of cancer, such as gastrointestinal stromal tumors (GIST), the primary mutation of D816V is a rare event. However, after cancer treatment, secondary mutation of D816V happens frequently, the selective and resistance advantage of D816V mutation leads to high recurrence in GIST patients.

Together with dependence receptor theory, we have proven that D816 mutation abolishes the pro-apoptotic activity of c-Kit, which leads us to reconsider the role of D816 mutation in cancers. We propose that D816 mutation renders tumor cells resistant to cell death by means of another mechanism, *i.e.* the silencing of c-Kit pro-apoptotic activity. Indeed, our experiments have shown that D816V mutation prevents cells from apoptosis induced by c-Kit. It should be noticed that D816V mutation blocks the c-Kit binding and activating of caspase-9 as well as MOMP, indicating that D816V mutation inhibits the apoptotic downstream signaling pathway of c-Kit.

Even if most dependence receptors are cleaved by caspases, not all of their caspase cleavage bands can be detected by western blot. In the case of c-Kit, it is also difficult to detect its C-terminal caspase cleavage band without proteasome inhibitor, because the small caspase cleavage band (~18kDa) is fast degraded. For this reason, we treated cells expressing either endogenous or induced c-Kit by using proteasome inhibitor MG132 or Bortezomib. After only 1 or 2 hours treatment, an accumulation of non-degraded caspase cleavage fragment appeared. It should be noticed that during the 1 or 2 hours treatment of proteasome inhibitor, caspase activity inside cells were not changed, meaning that the proteasome inhibitor only protected the caspase cleavage fragment from degrading, rather than causing caspase activation and c-Kit cleavage by themselves. Besides, when we

treated c-Kit expressing cells M2Ge with caspase activator (such as cIAP and XIAP inhibitor BV6, or direct caspase-3 activator PAC1), the amount of c-Kit caspase cleavage fragment was increased, confirming that the small band we detected was generated by caspase cleavage.

Future research should focus on deeply studying how is the caspase cleavage of c-Kit related to its pro-apoptotic activity. In particular, the following aspects should be developed:

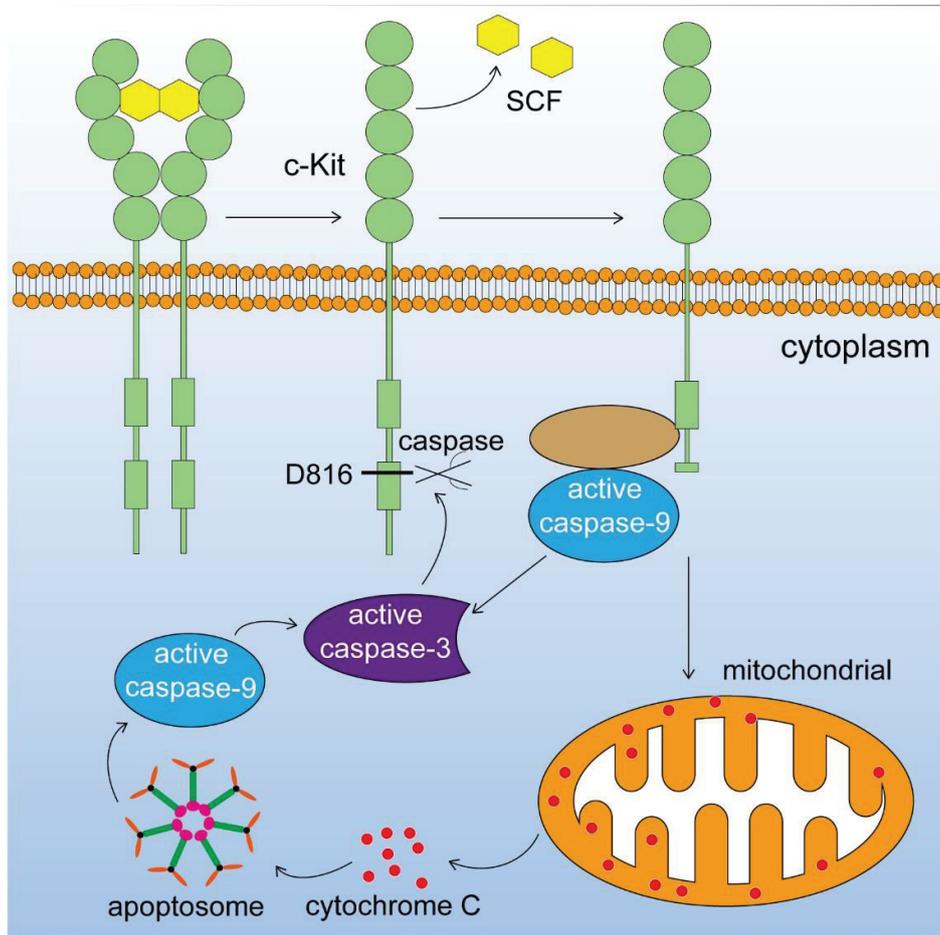
- 1) It has already been indicated that c-Kit caspase cleavage allows its binding and activating of caspase-9. But we have no idea yet if they bind directly or indirectly through other co-partners. Perhaps something can be learnt from another dependence receptor, the Sonic Hedgehog receptor Patched. It has been reported that Patched triggers apoptosis through the recruitment of a caspase-activating complex, called “dependosome”, which includes the Patched intracellular domain after caspase cleavage, adaptor proteins DRAL and TUCAN, and the apical caspase-9. After interaction, Patched recruits the ubiquitin ligase NEDD4, leading to caspase-9 ubiquitination and activation (Fombonne et al., 2012). Therefore, we can also further study whether c-Kit recruits a complex that includes caspase-9 and other co-partners, and whether c-Kit recruits some ubiquitinases that might activate caspase-9. Of course, other possible mechanisms concerning caspase-9 binding and activation should also be well studied. Besides, whether c-Kit membrane anchored part after caspase cleavage can bind other apoptotic partners and trigger alternative apoptotic signaling remains to be a question.
- 2) Besides, concerning the small cleavage fragment (from D816 to C-terminal end), whether or not it plays a role in the apoptotic signaling pathway should be well studied. For the other receptors which are both DRs and RTKs, their caspase cleavage fragments are the main apoptotic triggers. Taken TrkC as an example, Ichim G et al. performed a yeast two-hybrid screen with the TrkC KF as bait and a mouse embryonic complementary DNA library as prey, by which they found that the caspase cleavage fragment of TrkC interacts with Cobra1 (a putative cofactor of BRCA1), which shuttles TrkC cleavage fragment to the mitochondrial, where it promotes Bax activation, cytochrome c release, and apoptosome-dependent apoptosis (Ichim G et al., 2013). Thus, study should be made in the future to figure out whether the small caspase cleavage fragment of c-Kit can bind to any apoptotic

- partners by using similar screening method and whether this small fragment functions on mitochondrial or others cellular organelles.
- 3) The relation of the two caspase cleaved parts of c-Kit, *i.e.* the membrane anchored part and the small C-terminal fragment, is worth attention. It will be interesting to study whether they form a dimer after cleavage, and whether this dimer is mediating apoptosis signaling, such as recruiting a complex containing caspase-9. On the other hand, we cannot exclude the possibility that the small C-terminal fragment of c-Kit could act as an inhibitor to the pro-apoptotic function of membrane-anchored part of c-Kit. This could explain why c-Kit needs to be cleaved and eliminate D816 to C-terminal end part to trigger apoptosis.
  - 4) Furthermore, as to other receptors that are both RTK and DR (such as MET, RET and TrkC), they all have two caspase cleavage sites, generating a cleavage fragment that is essential for their pro-apoptotic activity. For example, the unbound TrkC undergoes cleavage at two intracytoplasmic sites by caspase-like proteases, leading to the release of the TrkC 496–641 fragment (Tauszig-Delamasure et al., 2007). Thus, whether or not c-Kit has additional caspase cleavage sites that are important for its pro-apoptotic signaling pathway could be studied in the future.
  - 5) Additionally, even though we have shown that the D816V mutated c-Kit could not be cleaved by caspase-3, it is not very strict to say that D816 is the direct caspase cleavage site. Because it is also possible that D816 site mutation could cause a structural change, which hides other possible cleavage sites from caspases. In particular, we are not very clear about the 3D structure of c-Kit or c-Kit intracellular-fragment in cells or in solution after D816 mutation. Thus, we just propose that D816 site is a possible caspase cleavage site. But at least, we are sure that D816V mutation inhibits caspase cleavage on c-Kit, either because of direct caspase cleavage site mutation or indirect structural changes following mutation.

## 6.7 c-Kit pro-apoptotic signaling pathway

Taken together all the results, we propose a possible c-Kit pro-apoptotic signaling pathway, described in Figure 6.3. Following our theory, in the absence of its ligand SCF, c-Kit is cleaved by caspases at D816 site. After the cleavage, c-Kit recruits co-partners and form a complex including caspase-9, where caspase-9 is activated. Then caspase-3 is activated either directly by active caspase-9 or through mitochondrial dependent

intrinsic apoptotic pathway. The increased active caspase-3 in turn cleaves more c-Kit on D816 site, resulting in an apoptotic positive feedback loop.



**Figure 6.3** c-Kit pro-apoptotic signaling pathway

Even though we have proven that c-Kit is indeed a member of dependence receptor and a pro-apoptotic signaling pathway model has been proposed, the detailed mechanism for this apoptotic pathway is not yet totally known. Until now, we have shown that the caspase cleavage site (D816) is essential for the apoptotic pathway. One of the possible mechanisms is that c-Kit, after caspase cleavage, binds and activates caspase-9. However, we are not clear about how c-Kit binds and activates caspase-9, i.e., if it is a direct binding or there is a co-partner to mediate this binding.

Except for caspase-9, we are also interested to find out other pro-apoptotic partners involved in c-Kit induced cell death. For this purpose, we plan to perform a CRISPR/Cas9-based screening, with a library of 19,050 target genes and 123,411 small guide RNAs (sgRNAs) (~6 sgRNAs per gene). Basically, stable cell lines inducible for kinase mutated c-Kit will be infected with a lentiviral sgRNA library (Sanjana et al., 2014). Lentiviral infection will be performed in order that, after puromycin selection, every stable inducible cell will have one gene knocked out by CRISPR/Cas9 technique. After induction of kinase mutated c-Kit overexpression, only cells which lost the genes important for c-Kit apoptotic pathways will survive. Thus, after sgRNAs amplification and high throughput sequencing of the resistant cells, we will have a group of candidate genes possibly important for the downstream apoptotic signaling of c-Kit. Later, as to the possible candidates, each of them will be separately knocked down in c-Kit stable inducible cells to find out the silence of which will prevent c-Kit induced apoptosis.

Besides the CRISPR/Cas9-based screening, other screening techniques, allowing deciphering of c-Kit pro-apoptotic pathways, will also be applied, such as proximity-dependent biotinylation screening. In this screening, c-Kit will be fused with BioID2 protein, which belongs to biotin protein ligases that are the enzymes existing in many organisms and can attach biotin to a lysine. Thus, the endogenous proteins that have juxtaposition to the BioID2-fused c-Kit will be biotinylated. The biotinylated proteins will be captured by agarose beads or magnetic beads coated with streptavidin, which has highest binding affinity to biotinylated proteins. Further, mass spectrum analysis of the captured proteins will determine their identity (Kim et al., 2017). Alternatively, a proteomic mapping will be obtained by fusing c-Kit to the engineered peroxidase APEX2. After treatment of living cells with hydrogen peroxide ( $H_2O_2$ ), in presence of biotin-phenol, endogenous proteins close to c-Kit-APEX2 protein will be tagged by biotinphenoxy radical, allowing their purification with streptavidin beads and subsequently identification by mass spectrometry (Lam et al., 2015). In this way, we can possibly find some binding partners of c-Kit.

Regardless of the fact that some DRs, such as DCC and Patched, recruit a complex “dependosome” containing caspase-9 after caspase cleavage (Forcet et al., 2001; Mille et al., 2009b), the initiator recruited caspase is not always necessary to be caspase-9. Indeed, it has also been shown that integrins, as DRs, trigger apoptosis through recruitment of caspase-8 (Stupack et al., 2001). Since caspase-8 is the initiator for extrinsic apoptosis pathway that initiates apoptosis involving transmembrane receptor-

mediated interactions. It could also be possible that c-Kit somehow binds and active caspase-8, triggering extrinsic apoptosis as well.

Furthermore, about the mechanism how SCF inhibits the pro-apoptotic activity of c-Kit, questions remain to be answered. Does SCF function through directly inhibiting the caspase cleavage on c-Kit or through disturbing the binding of c-Kit to its pro-apoptotic partner (such as disturbing the formation of a complex containing caspase-9 and other partners)? Besides, the SCF inhibition of c-Kit pro-apoptotic activity, is it through c-Kit conformation change or through other non-structural changes? Future research is need to answers all these questions.

## **6.8 Therapeutic strategy targeting c-Kit related cancers**

### **6.8.1 SCF expression and cancer**

SCF-mediated c-Kit signaling pathway plays important roles in mediating angiogenesis, migration, cell survival and proliferation (Matsui et al., 2004; Lennartsson et al., 2012). The morphological and immunohistochemical analysis revealed that several human tumor tissues, such as human cervical adenosquamous carcinomas (Martinho et al., 2008), Merkel cell carcinoma (Krasagakis et al., 2009), renal tumor (Horstmann et al., 2012) and rat cholangiocarcinogenesis (Mansuroglu et al., 2009) simultaneously express both SCF and c-kit proteins.

Since we have proven that c-Kit is a dependence receptor, whose pro-apoptotic activity is regulated by ligand SCF, upregulation of SCF not only increases c-Kit kinase activity, but also inhibits c-Kit pro-apoptotic activity, favoring tumor progression. Concerning ligands of other DRs, upregulation of ligands including Netrin-1, NT3, and Semaphorin 3E, etc (Fitamant et al., 2008; Luchino et al., 2013; Mancino et al., 2011) are often detected in different type of cancers and their upregulation could be correlated to metastasis. This phenomenon makes DR ligands as putative biomarker for the prognostics and potential therapeutic targets. Thus, we wonder if SCF is also possibly upregulated in some types of cancer.

Not many studies indicate that SCF expression is increased in cancers, since usually the focus is on upregulation of c-Kit level. However, there are still some reports indicating that there is a significantly increased expression of SCF in hepatocellular carcinoma

(HCC), and that SCF is an independent prognostic biomarker for overall and relapse-free survival of patients with HCC (Wang et al., 2014). Some other reports show that HIF-1 $\alpha$  is a key regulator of SCF expression in breast cancer cells. Hypoxia and epidermal growth factor receptor signal coexisted in the tumor microenvironment and might promote angiogenesis through HIF-1 $\alpha$ -mediated upregulation of SCF and other angiogenic factors (Han et al., 2008). Besides, HIF-1 $\alpha$  promotes the development of pancreatic ductal adenocarcinoma (PDAC) by trans-activating SCF under hypoxia (Gao et al., 2015).

### **6.8.2 c-Kit-EC (extracellular domain) and conditional medium**

In order to block SCF/c-Kit binding, we have used c-Kit-EC, which has been reported to interact with SCF (Philo et al., 1996) and act as a trap able to interfere with the binding between SCF and the membrane-bound c-Kit (Lev et al., 1992; Wypych et al., 1995; Dahlen et al., 2001). Besides, this is a more therapeutically relevant approach to block SCF/c-Kit binding than knocking SCF down by siRNA, even though our results indicated that knocking SCF down by siRNA could trigger apoptosis as well.

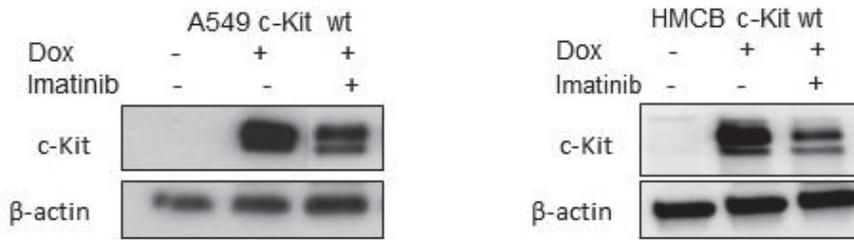
Actually, the physiological c-Kit-EC can also be observed in secretion medium where c-Kit positive cells are cultured, which is a result of extracellular proteolytic cleavage of c-Kit (Broudy et al., 1994; Turner et al., 1995). In data that is not shown, we have observed that, when c-Kit positive cells were treated with TAPI-1 (TNF-alpha protease inhibitor I), the c-Kit-EC secretion was inhibited. Since TAPI-1 is a drug for the inhibition of MMPs (matrix metalloproteinases) and TACE (TNF- $\alpha$  converting enzyme/ADAM17), it is possible that c-Kit is cleaved by MMPs or TACE, creating c-Kit-EC. Interestingly, in some cells (such as HMCB) that are induced for overexpression of either wild-type or kinase mutated c-Kit, when we treated cells with TAPI-1, we could detect an increased amount of full-length c-Kit protein, as a result of proteolytic cleavage inhibition. The increase of wild-type or kinase mutated c-Kit triggered even stronger apoptosis comparing to cells without TAPI-1 treatment, further proving that c-Kit acts as a dependence receptor with pro-apoptotic activity.

Conditional medium containing c-Kit-EC was also obtained from non-serum medium culturing cells inducible for c-Kit-EC expression. There are several advantages of using conditional medium containing c-Kit-EC. First, we can use c-Kit-EC from conditional medium to treat any c-Kit positive cells, without necessarily making them all stable inducing cells overexpressing c-Kit-EC by themselves, which is also time saving. Second,

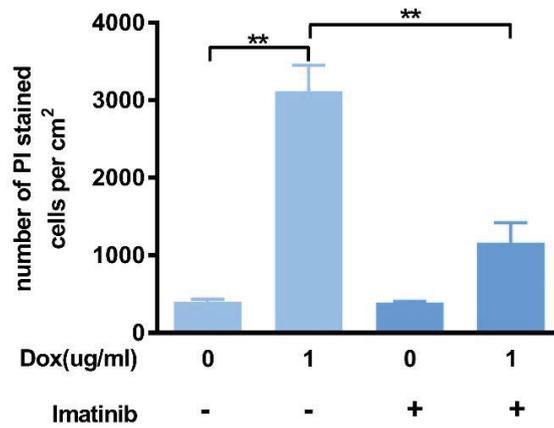
the conditional medium containing c-Kit-EC can be concentrated by using centrifugal filter Centricon® for enhancing c-Kit-EC concentration, thus smaller dose of conditional medium is needed to reach the same effect. Third, conditional medium can be stored at -80°C and kept stable and effective for a long period.

### **6.8.3 The effect of imatinib on c-Kit**

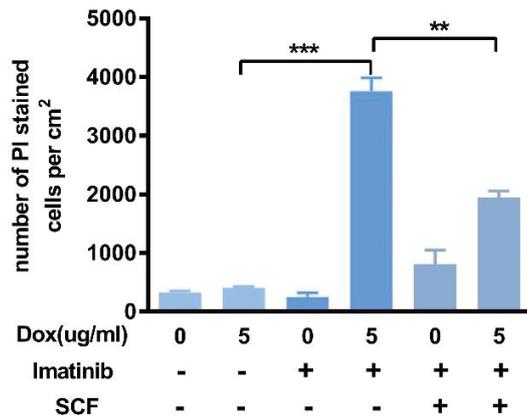
Imatinib is a commonly used TKI targeting c-Kit. Some interesting phenomenon was observed during imatinib treatment to our stable inducible cell lines expressing c-Kit. When imatinib was added to cells overexpressing c-Kit, a decrease of c-Kit protein amount was detected (Figure 6.4). It has already been reported that imatinib can down-regulate c-Kit by inducing c-Kit internalization and its subsequent lysosomal degradation (D'allard et al., 2013). Interestingly, by downregulating c-Kit, imatinib also partially inhibited apoptosis induced by c-Kit in HMCB stable cell lines (Figure 6.5). This phenomenon is on the contrary to classic view of c-Kit only as a receptor tyrosine kinase, but it meets our proposal that c-Kit is a dependence receptor with pro-apoptotic activity. The downregulation of c-Kit by imatinib impaired the apoptosis induced by overexpressed c-Kit. The effect of imatinib also depended on cell types. In cell lines sensitive to overexpressed wild-type c-Kit (such as HMCB), the effect of imatinib trended to be apoptosis inhibition by c-Kit downregulation (Figure 6.5). However, in some other cell lines where overexpressed wild-type c-Kit did not kill cells by itself (such as A549), co-treatment of imatinib could somehow increase cell death (Figure 6.6), probably because imatinib inhibited the kinase activity in these cells, making c-Kit “kinase activity and pro-apoptosis activity balance” favor apoptosis (even the downregulation of total c-Kit protein still existed). The cell death induced by co-treatment of imatinib could be inhibited by adding ligand SCF (Figure 6.6). Thus, imatinib actually has two faces on the function of c-Kit: competing the ATP binding site on c-Kit to directly inhibit kinase activity; downregulating receptor c-Kit which impairs both the kinase and pro-apoptotic activity inside cells. The final effect of imatinib on c-Kit positive cells depends on the cell types and the c-Kit “kinase activity and pro-apoptosis activity balance” inside the cells. (Figure 6.7)



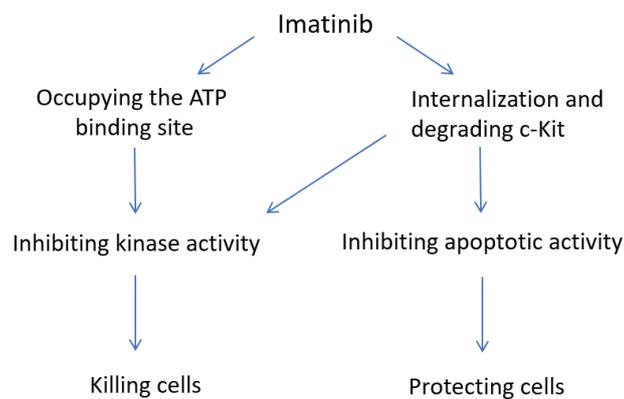
**Figure 6.4** Imatinib degrades c-Kit in stable inducible cell lines. A549 and HMCB stable inducible cell lines were treated with 1  $\mu$ g/ml doxycycline for wild-type c-Kit induction; 1  $\mu$ M imatinib was added at the same time with doxycycline. Dox: doxycycline



**Figure 6.5** Imatinib inhibits c-Kit induced cell death by degrading c-Kit in in HMCB stable inducible cells. c-Kit overexpression in HMCB stable cells triggered cell death, while the presence of 1  $\mu$ M imatinib inhibited cell death induced by c-Kit. Cell death was analyzed by propidium iodide (PI) staining 36 hours after doxycycline and imatinib treatment, using Incucyte Zoom instrument. Dox: doxycycline. \*\*, p < 0.01.



**Figure 6.6** Co-treatment of imatinib enhances cell death by inhibiting kinase activity in A549 stable inducible cells, which can be inhibited by adding ligand SCF. Wild-type c-Kit overexpression in A549 was not able to induce cell death. When A549 stable cells were co-treated with 5  $\mu$ M imatinib, the pro-apoptotic activity of c-Kit was released and cell death was enhanced. Additional treatment with 100ng/ml SCF was able to inhibit the cell death triggered by imatinib co-treatment. Cell death was analyzed by propidium iodide (PI) staining 36 hours after doxycycline and imatinib treatment, using Incucyte Zoom instrument. Dox: doxycycline. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



**Figure 6.7** Imatinib functions on c-Kit, leading to a balance between killing cells and protecting cells. Whether a c-Kit expressing cell undergoes survival or death after imatinib treatment depends on the c-Kit “kinase activity and pro-apoptosis activity balance” inside the cell.

#### 6.8.4 Imatinib treatment and novel therapeutic strategy

Imatinib (Gleevec) is used as the first line drug targeting c-Kit related disease, acting as an inhibitor of c-Kit kinase activity. However, some mutations, such as D816V mutation, lead to resistance against imatinib. We have used imatinib to treat different c-Kit positive

cell lines, while each cell line showed different sensitivity to imatinib. In some cell lines, such as M2Ge, even if they were c-Kit expressing cells, imatinib was not sufficient to kill them unless the doze reached very high. However, when we knocked down ligand SCF, the co-treatment of imatinib induced apoptosis in a short time. Thus, we propose that we can use novel strategy – combination of interfering SCF/c-Kit binding and kinase inhibitor treatment – to treat some c-Kit related disease where c-Kit is not mutated at D816 site. By this strategy, we both inhibit the kinase activity and release the pro-apoptotic activity of c-Kit, results in better killing effect. In the case of eliminating cells containing D816 mutated c-Kit, kinase inhibitor which targets the downstream RTK signaling pathways, such as dasatinib, can be co-treated.

As to interfering SCF/c-Kit interaction, the c-Kit-EC that we produced has already shown significant efficacy. However, antibody that blocks SCF/c-Kit binding should have similar effect of achieving apoptosis in c-Kit expressing cells, while it might be a better clinical method. Blocking antibody targeting ligands of other DRs has already been applied in experiments and even clinical research. For example, a humanized monoclonal antibody disrupting netrin-1- receptors interaction has been developed in our laboratory and is currently assessed in early clinical trial (<https://clinicaltrials.gov/ct2/show/NCT02977195>). Thus, antibody targeting and blocking SCF/c-Kit interaction can be developed in the future, which will possibly be used in the novel therapeutic method in combination with TKIs co-treatment.

## **6.9 Conclusion of discussion**

At the beginning of our project, we have noticed that, in some reports, c-Kit is downregulated in some types of cancer and its downregulation is related to poor prognosis, which is on the contrary to its role as a RTK. However, our results demonstrate that c-Kit, except for being a RTK, is also a member of dependence receptor, triggering apoptosis in the absent of its ligand SCF. Following caspase cleavage at D816 site, c-Kit is able to bind and activate caspase-9, inducing apoptosis signaling.

c-Kit related disease has long been treated with tyrosine kinase inhibitors (TKIs) such as imatinib, while the resistance against TKIs occurs frequently. One of the major mutations that causes resistance is the mutation on D816 site of c-Kit. Besides, it has been reported that during the cancer progression or treatment, there is an accumulation

of D816 mutation (Antonescu et al., 2005). Moreover, in some types of cancer, such as acute myeloid leukemia, D816V mutation is related to poor prognosis (Cairolì et al., 2006). The classic view to explain this role of D816 mutation is that it results in constitutive kinase activity that favors cancer progression and drugs like imatinib lose the ability to compete the ATP binding site for inhibiting the kinase signaling. On the other hand, our results have shown that c-Kit is cleaved by caspases on D816 site, which is essential for its pro-apoptotic activity. The mutation of D816 site prevents apoptosis induced by c-Kit. Therefore, we propose an alternative explanation for role of the D816 mutation. Cancer cells with D816 mutation on c-Kit lose the c-Kit pro-apoptotic activity, resulting in growth advantage and resistance against TKIs treatment. Thus, D816 mutation can accumulate in cancer development or treatment. Furthermore, we have proven that the presence of ligand SCF is able to inhibit the pro-apoptotic activity of c-Kit, while by blocking SCF/c-Kit interaction, we are able to induce cell death in c-Kit expressing cells. Finally, we propose a novel therapeutic strategy, combining TKIs treatment with the interference of SCF/c-Kit binding to unleash c-Kit pro-apoptotic activity and achieve a better therapeutic effect.

# V. Methods and Materials

## Cell Lines, Transfection Procedures, Reagents

Human lung A549 and colon HCT116 cancer cell lines were cultured in DMEM medium (Life Technologies) containing 10% foetal bovine serum. Human neuroblastoma IMR32 cell line was cultured in RPMI 1640 + Glutamax medium (Life Technologies) containing 10% foetal bovine serum. Human melanoma HMGB, M2Ge and XPC cell lines were cultured in DMEM + Glutamax supplemented with pyruvate (Life Technologies). For RNA interference experiments, cell lines were transfected using lipofectamine RNAiMAX reagent (Life Technologies). For plasmid transfection, A549 and IMR32 cell lines were transfected using lipofectamine Plus reagent (Life Technologies); HCT116 cell line was transfected using jet prime (PolyPlus-Transfection); HMGB cells were transfected with FuGENE HD (Promega). All transfections were performed according to the manufacturer's protocol.

For activating c-Kit kinase activity or inhibiting c-Kit pro-apoptotic activity, we have used recombinant human SCF (hSCF) from different companies. Recombinant SCF protein was obtained from R&D systems (E.coli-derived) or Cell Signaling (293T-derived). The recombinant SCF that we used can be catalyzed into two groups: those produced in E. coli (*Escherichia coli*) bacterial (such as hSCF, 255-SC, R&D System) and those produced in human cells (such as 293 cells) (such as hSCF, #8925, Cell Signaling Technology). Even though they are both recombinant human SCF with same amino acids, they migrated to very different molecular weight positions in western blot. Those produced in bacterial migrated to around 18 kDa while those produced in human cells migrated to around 30 to 40 kDa. Actually, both of the recombinant hSCFs contain Glu26-Ala189 of hSCF, which corresponds to core function part of soluble SCF after proteases cleavage. However, even the soluble SCF contains only 163 amino acid, it should migrate to around 30 to 40 kDa in western blot because SCF is glycosylated. It means that the recombinant hSCF produced in bacterial lacks glycosylation (and maybe some other post-translational modification (PTM) as well), while recombinant hSCF from human cells contains these PTMs. This also corresponds to our results that recombinant hSCF produced in human cells was more effective than those from bacterial. For example, when recombinant hSCF was used to inhibit c-Kit pro-apoptotic activity, much less recombinant hSCF from human cells (Cell Signaling) was needed to reach the same effect as that from bacterial (R&D System), indicating that PTMs are important to the function of SCF.

## Generation of stable cell lines

To investigate whether c-Kit and its mutants are able to induce apoptosis, we would like to overexpress c-Kit and its mutants in cells. Usually two different methods can be used, either transient transfecting c-Kit into cells or creating stable cell lines expressing c-Kit under induction. The transient transfection method has several disadvantages: the transfection method itself is somehow toxic to the cells, which causes the immediate cell death test less reliable; the c-Kit protein expression level after transient transfection is often much too high comparing to physiological c-Kit protein level, making the cell models less appropriate for studying c-Kit function in physiology. Therefore, we decided to make stable cell lines that express c-Kit or its mutants under induction.

For the generation of inducible stable cell lines, we used a sleeping beauty-based vector allowing the doxycycline inducible expression of c-Kit or its mutants (details in the following paragraph). Using this method, we can induce the expression of c-Kit or its mutants, just adding appropriate dose of doxycycline, which was not toxic to cells at the concentrations we used. Over the benefit to induce c-Kit expression on-demand, the other important advantage of this method is the ability to control the dose of doxycycline added, making the c-Kit protein expression level comparable to physiological c-Kit protein level (see Supplementary Figure 1C). This feature allows us to obtain better stable inducible cell models to study c-Kit functions under more physiological conditions.

Considering the details of generating inducible stable cell lines, flag-tagged human c-Kit coding sequence was cloned at Sfil sites in the pITR plasmid, a sleeping beauty-based vector allowing the doxycycline inducible expression of c-Kit or c-Kit-EC, as well as the constitutive expression of rtTA protein, puromycin resistance gene and green fluorescent protein (GFP), expressed under the control of the mouse phosphoglycerate kinase (PGK) promoter. Alternatively, c-Kit was cloned in pSB-tet plasmids, a second-generation of sleeping beauty-based vectors, containing an artificial promoter (RPBSA) to drive expression of rtTA protein, antibiotic resistance gene and fluorescent protein (Kowarz et al., 2015). A549, IMR32, HMCB and HCT116 cell lines were co-transfected with c-Kit-pITR and a sleeping beauty transposase expression vector (SB100X). pITR vector was a generous gift of Prof. Rolf Marschalek (Goethe-University of Frankfurt, Germany). pSBtet-GH was a gift from Eric Kowarz (Addgene plasmid # 60498). Stable cell clones were selected for 5 days in puromycin or hygromycin-containing medium. Then wt and mutated c-Kit stable cell lines were sorted by flow cytometry to selected GFP positive cells, which

are the stable inducible cells. All the stable inducible cell lines were further tested for c-Kit or c-Kit-EC expression in response to doxycycline.

Stable cell line constitutively expressing the intracellular region of c-Kit were generated by lentiviral infection, using self-inactivating HIV-1-derived vector (pWPXLd), encoding c-Kit (540-976 region) under the control of Human Elongation Factor-1 alpha (EF-1 Alpha) promoter. For the generation of stable cell line knocked-out for c-Kit or caspase-3 and -9, different small guide RNAs (sgRNA) were designed using the sgRNA designer tool (CRISPRko), available at <http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design> site. BsmBI linker were added to sgRNAs and the oligonucleotides were then annealed by incubation for 3min at 90°C and 15min at 37°C and ligated in plentiCRISPR v2 vector (Sanjana et al., 2014). Insertion of sgRNA was confirmed by sequencing. The use of plentiCRISPR v2, which is constructed around a 3rd generation lentiviral backbone, allows for the simultaneous infection/transfection of the vector for the expression of Cas9 and gRNA and for selection for puromycin resistance. Lentiviral vectors were a generous gift of Carine Maise (INRA UMR754, Lyon, France).

### **Quantitative RT-PCR**

Total RNAs from cancer cell lines were extracted using NucleoSpin® RNAII Kit (Macherey Nagel) according to manufacturer's protocol. Total RNAs from xenograft tumours were extracted by disrupting tissues with MagNALyser instrument (Roche Applied Science). RT-PCR reactions were performed with PrimeScript RT Reagent Kit (Takara). One micro gram total RNA was reverse transcribed using the following program: 37°C for 15min and 85°C for 5sec. For expression studies, the target transcripts were amplified in LightCycler®2.0 apparatus (Roche Applied Science), using the Premix Ex Taq probe qPCR Kit (Takara), according to manufacturer instructions. Expression of target genes was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA binding protein (TBP) and phosphoglycerate kinase (PGK) genes, used as house keeping genes. The amount of target transcripts, normalized to the housekeeping gene, was calculated using the comparative CT method. A validation experiment was performed, in order to demonstrate that efficiencies of target and housekeeping genes were approximately equal. The sequences of the primers are available upon request.

## Western Blotting Analysis

For immunoblotting analysis of c-Kit proteins, phospho-ERK and phospho-c-Kit, and active caspase-3 stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% or 2% (for caspase activation) serum. Cells were then lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor (Sigma). For immunoblotting analysis of engrafted tumour cells, after sacrificing the mice, tumours were grinded and sonicated in RIPA lysis buffer in the presence of protease inhibitor mixture (Roche Applied Science).

Lysates were incubated 1h at 4°C and cellular debris were pelleted by centrifugation (10.000g 15' at 4°C) and protein extracts (20-50µg per lane) were loaded onto 4-15% SDS-polyacrylamide gels (Biorad) and blotted onto nitrocellulose sheets using Trans-Blot® Turbo™ Transfer System (Biorad). Filters were blocked with 10% non-fat dried milk or 10% BSA in PBS/0.1% Tween 20 (PBS-T) for 1 hour and then incubated over-night with mouse monoclonal α-c-Kit (dilution 1:2000, clone # 47233, R&D systems), rabbit polyclonal α-c-Kit (1:2000, Enzo Life Science), goat polyclonal α-SCF (1:1000, R&D systems), mouse monoclonal α-β-actin (1:1000, clone C4, HRP-conjugated, Santa Cruz Biotechnology), rabbit polyclonal α-HA (1:2000, Sigma), rabbit polyclonal α-caspase-3 (1:1000, Cell Signalling Technology), mouse monoclonal α-phospho-tyrosine (1:1000, clone PY20, Santa Cruz Biotechnology), mouse monoclonal α-phospho-ERK1/2 (1:1000, Sigma) and rabbit polyclonal α-ERK1/2 (1:1000, Sigma) antibodies. After three washes with PBS-T, filters were incubated with the appropriate HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch, Suffolk, UK) for 1h. Detection was performed using West Dura Chemiluminescence System (Pierce). Membranes were imaged on the ChemiDoc Touch Imaging System (Biorad).

## Membrane staining of wild-type or mutated c-Kit

IMR32 c-Kit stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% serum. Cells were washed and detached using 0.53mM ethylenediaminetetraacetic acid (EDTA) in PBS.  $1 \times 10^5$  cells were collected for each condition and washed twice with 1ml PBFA (PBS-2% Foetal Bovine Serum-0.1% NaN<sub>3</sub>). Then cells were incubated with antibody anti-c-Kit conjugated with Phycoerythrin (PE)

(CD117-PE, human, clone: AC126, Miltenyl Biotec) in 100ul PBFA buffer for 1 hour at 4°C. After being washed twice again by PBFA, cells were re-suspended in 300µl cold PBS and were then assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur (BD Biosciences).

### **In Vitro Transcription/Translation and Caspase Cleavage Reactions.**

Purified caspase-3 were a generous gift from Guy Salvesen (The Burnham Institute, La Jolla, CA). In vitro transcription/translation and incubation with caspases-3 were performed as described previously (Mehlen et al., 1998).

### **Cell Death Assay**

For propidium iodide (PI) staining, stable inducible cell lines were seeded in 96-well-plates at density of  $1.5 \times 10^3$  cells/well (A549) or  $3 \times 10^3$  cells/well (IMR32) or in 12-well-plates at density of  $1 \times 10^5$  cells/well (HMCB and HCT116). The following day, cells were treated with doxycycline and 0.33µg/ml PI in serum-free (A549) or serum-poor (2%) medium (IMR32, HMCB and HCT116) and the plates were transferred into Incucyte Zoom instrument (Essen Bioscience). Images of living cells and PI-stained dead cells were taken every two or three hours and PI-stained cell numbers were calculated.

For AnnexinV/PI double staining, cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMCB and HCT116) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 24 hours by doxycycline. Then cells were collected, washed with PBS and incubated for 15 min on ice in Annexin V buffer containing allophycocyanin (APC)-conjugated Annexin V and PI (BioLegend). Annexin V-positive cells were then assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur (BD Biosciences).

For DNA fragmentation analysis (SubG1), HMCB or HCT116 cell lines were plated onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well. The following day, cell medium was replaced by low-serum (2%) medium and cells were treated with doxycycline for 24 hours, 48 hours or 72 hours. For experiments combining SCF silencing and Imatinib treatment, M2Ge cells were plated onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well 24 hours before to be transfected with siRNAs. 24 hours after transfection, transfected cells were treated

with Imatinib (Sigma) in non-serum medium for 24 hours. Then cells were collected and fixed in 70% ethanol and incubated at -20°C for at least one night. Further, cells were washed twice with PBS and incubated for 15 min at room temperature in PBS containing 2 mg/ml RNase A and 40µg/ml PI. DNA content was evaluated by fluorescence activated cell sorting (FACS) analysis on a FACScalibur.

Caspase-3 activity assay was performed as described previously (Paradisi et al., 2009). Briefly, stable inducible cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMGB and HCT116) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 24 hours by doxycycline. Cells were collected and caspase-3 activity assay was performed using the Caspase 3/ CPP32 Fluorometric Assay Kit (BioVision), according to the manufacturer's instructions. Caspase activity (activity / min / mg of protein) was calculated from a 1h kinetic cycle reading on a spectrofluorometer (405nm / 510nm, Infinite F500, Tecan).

TUNEL-PI double staining assay was performed using DeadEnd™ Fluorometric TUNEL System (Promega), according to manufacture protocol. Briefly, HCT116 cells were seeded onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well. 24 hours after, cell medium was replaced by low-serum (2%) medium and cells were treated with doxycycline for further 48 hours or 72 hours. Then, cells were collected and fixed in 5ml of 1% methanol-free formaldehyde for 20 minutes on ice. After washing twice with PBS, cells were permeabilized by ice cold 70% ethanol for 24 hours and then washed again twice with PBS. Finally, cells were stained with fluorescein-12-dUTP and PI and TUNEL staining and DNA content was assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur.

### **Discussion about Cell Death Assays**

In our project, various cell death tests were frequently used. One of our most important aim was to prove that c-Kit is able to induce cell death, especially through apoptosis. One single method of cell death test is usually not enough to define apoptosis, because of the overlaps between apoptosis and other types of cell death. Thus, the combination of different cell death assay methods is needed to study apoptosis.

Dead cells allow Propidium iodide (PI) to enter inside the cells while alive cells do not. Thus, PI staining is a method studying cell death. In our project, we used Incucyte Zoom instrument (Essen Bioscience), which is able to take images of living cells and PI-stained dead cells every two or three hours and then calculate the number of PI stained cells. The advantage of this experiment is that it allows us to view the dynamic changes of cell death caused by stimulus (such as c-Kit expression), which is also useful when we compare the cell death curves of different stimulus. The biggest disadvantage is that PI staining can't distinguish between apoptosis and necrosis since both of these two forms of cell death allow PI staining of DNA. Therefore, other types of cell death assays, especially those that can tell the difference between apoptosis and necrosis, should be applied.

AnnexinV/PI double staining analyzed by flow cytometry is a method capable of partially distinguishing between apoptosis and necrosis. After staining, the AnnexinV- / PI- cell are the alive cells, while the AnnexinV+/PI- cells are those undergo early apoptosis. However, the big shortcoming of AnnexinV/PI double staining is that we are not able to separate late apoptosis from necrosis, cause cells in both of these two cell death forms are AnnexinV+/PI+. Thus, even though AnnexinV+ cells are often considered as apoptotic cells, we can not exclude the possibility that some of them might be necrotic cells. The other disadvantage of this assay is that we are not able to see the dynamic changes of cell death caused by stimulus.

DNA fragmentation analysis (sub-G1 assay) is an assay that is able to exclude the cases of necrosis, cause inter-nucleosomal DNA fragmentation is one of the hallmarks of apoptosis, while it doesn't happen in necrosis condition. Even though necrotic cells can peak into sub-G1 area due to loosely packed DNA (which can reduce the fluorescence intensity and thus the G1 peak falls into sub-G1 area), the necrosis peak is very close to G1 peak whereas apoptotic peak will be far off from G1 peak. Thus, the apoptotic cells can be identified by stringent comparison with control cells. There are also some shortcomings of sub-G1 assay. First, if cells enter apoptosis from the S or G2/M phase of the cell cycle or if there is an aneuploid population undergoing apoptosis, they may not appear in the sub-G1 peak. Second, if the cell population is heterogenous, with different G1 peaks from different subpopulations, it is difficult to define which are the sub-G1 cells.

TUNEL-PI double staining analyzed by flow cytometry avoids the shortcomings of both Annexin/PI staining and sub-G1 assay. Actually TUNEL-PI staining is more like a combination of TUNEL staining and sub-G1 assay, solving the problem of sub-G1 that it

cannot detect some apoptotic cells from S or G2/M phase. TUNEL-PI staining can well define the apoptotic cells. The shortcoming is that the experiment procedure and analysis are more complicated and cost longer time than the other cell death assays. In our project, because the majority of our cells are in G0/G1 phase and the cell population is homogenous for G1 peak, sub-G1 assay is well appropriate and time-saving to replace TUNEL-PI double staining.

Caspase-3 activity test has also been performed either by Caspase3/ CPP32 Fluorometric Assay Kit (BioVision) (both caspase-3 and 7 activities are detected by a spectrofluorometer) or by western blot detecting both active and inactive caspase-3. Caspase-3 activity assay is based on the fact that both intrinsic and extrinsic apoptosis finally induce caspase-3/7 activity, while necrosis are classically considered unable to active caspase-3/7. Thus, this is also an assay to define apoptosis. Disadvantages also exist, one of them is that we have to choose several different time points to perform caspase-3 activity assay since caspase-3/7 activation is an early and dynamic process, which varies a lot at different time points after apoptosis is triggered.

Taken together the advantages and disadvantages of different cell death tests, it explains the reason why we needed several different types of cell death assays to study the cell death (particularly apoptosis) triggered by c-Kit. Besides, by using caspase inhibitors (such as Q-VD) or by knocking down caspases by CRISPR/Cas9 technique, we have further proven that c-Kit is able to induce apoptosis in cancer cells.

### **Immunoprecipitation (IP) and caspase-9 assay**

Stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% serum. Alternatively, 293T cells were transfected with both kinase-mutated or double-mutated c-Kit and caspase-9 plasmids, using Fugene HD (Roche Applied Science) and according to manufacturer instructions. Cell culture dish was placed on ice and cells were washed with ice-cold PBS (Phosphate buffered saline), and ice-cold lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 0.1% Nonidet P-40 (NP-40), 2 mM EDTA) was added on to the cells. Adherent cells were scraped off the dish then cell suspension was transferred gently into a microcentrifuge tube, sonicated and maintained at 4°C for 1 hour. Lysates were then centrifuged at 17,000 x g for 30 minutes at 4°C and the supernatants were transferred in a fresh tube kept on ice. Protein concentration was

measured and the same amount of protein was used for each sample. Lysates were incubated with 10ul of protein G-agarose beads for 30 min at 4°C with gentle rotation and then spun in microcentrifuge at 5,000 x g for 5 min at 4°C, and precleared supernatants were incubated with  $\alpha$ -flag antibody (1:100 dilution) overnight at 4°C under gentle rotation. Next, 50ul protein G-agarose beads was mixed with each sample and incubated for 2 hours at 4°C with gentle rotation. After the incubation, the mixture was then centrifuged, supernatant removed from the beads and discarded. Beads were washed with lysis buffer five times to remove non-specific binding. For each wash, beads were mixed gently with wash buffer, centrifuged at 4°C and the supernatant was then discard. After final wash, beads were resuspended in 50ul lysis buffer, and the elution/beads mixture was used for caspase-9 assay, using the Caspase-Glo 9 Assay Kit (Promega), according to the manufacturer's instructions. Luminescence, proportional to caspase-9 activity, was recorded on the Infinite F500 luminometer (Tecan). For western blot, lysates from 293T cells transfected with Caspase-9-HA and inducible kinase-mutated c-Kit were immunoprecipitated with  $\alpha$ -HA antibody (1:100, Sigma), incubated overnight at 4°C. After addition of protein G-agarose beads and incubation for 2 hours at 4°C, pulled-down proteins were eluted in laemmli sample buffer 2x. After incubation at 95C for 10 minutes and a brief spin, immunoprecipitated proteins were resolved by SDS-PAGE and pulled-down c-Kit was detected using mouse monoclonal  $\alpha$ -c-Kit antibody (1:2000, R&D systems).

### **Mitochondrial potential assay**

Stable inducible cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMCB) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 36 hours with doxycycline. Then cells were collected, washed with PBS.  $2 \times 10^5$  (HMCB) or  $4 \times 10^5$  (IMR32) cells for each sample were suspended in 200 $\mu$ l buffer containing JC-1 dye (Biotium). After incubation at 37 °C for 15 minutes, stained cells were centrifuged at 800 x g for 5 min at room temperature and supernatant was removed completely without disturbing the cell pellet. Afterwards, cell pellet was resuspended in 1 ml PBS for washing, then centrifuged at 800 x g for 5 min at room temperature and supernatant was removed again. Washing step was repeated once. Later, cell pellet was resuspended by pipetting in 50 $\mu$ l PBS buffer containing 1  $\mu$ g/ml DAPI. The final stained cells were analyzed by using NucleoCounter NC-3000 (Chemometec).

### **Conditional medium production**

HCT116 stable cells inducible for c-Kit-EC were plated in 75 cm<sup>2</sup> dishes (2.5\*10<sup>6</sup> cells/dish). The following day, cell medium was replaced with 8ml serum-free medium and cells were treated for 24 hours with or without doxycycline. Collected cell media were centrifuged at 1200Xg for 5 minutes and supernatant was recovered as c-Kit-EC or control conditional medium.

### **Animal model**

Five-week-old (20-22 g body weight) female SCID mice were obtained from Charles River animal facility. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. Stable HMCB and HCT116 cell lines were implanted by subcutaneous injection of 5\*10<sup>6</sup> cells in 100 µl of growth factors reduced matrigel (Corning) diluted in 100µl of PBS into the right flank of the mice. When tumours reached 100 mm<sup>3</sup> in approximately one week after injection, 1mg of doxycycline in 200µl or an equal volume of PPI water for control group was intraperitoneal injected daily for ten days. Tumour sizes were measured with a calliper. The tumour volume was calculated with the formula  $v = 0.5 \times (l \times w^2)$ , where v is volume, l is length, and w is width. All experiments were performed in accordance with relevant guidelines and regulations of animal ethics committee (Authorization no CLB-2015-004; accreditation of laboratory animal care by CECCAP, ENS Lyon-PBES, France).

Several xenograft mice models were used in our project to prove that c-Kit and c-Kit-EC have tumor suppressive effect. All the xenografted cells were stable inducible cells, where doxycycline is needed to induce the expression of c-Kit or c-Kit-EC. This was the first time that we have used doxycycline injected mice model, so that we needed to explore the experiment protocol. From the beginning, we intraperitoneally injected 1mg doxycycline each time to one mice and we kept this dose of doxycycline for all the experiments. But later, we found that each mouse could be injected from 0.1mg doxycycline to have an induction of target protein expression. So that in the future doxycycline induced mice models, we can use between 0.1mg to 1mg dose of doxycycline

injection to each mouse. Concerning the frequency of doxycycline injection, we have performed western blot to analyze the stability of one single doxycycline injection effect. As shown in Supplementary Figure 3, c-Kit expression could be detected in 24 hours after doxycycline injection, while not after 48 hours. Thus, the effect of doxycycline injected was quite limited in time. Based on this, we have decided to inject doxycycline every 24 hours for 10 days in total in each experiment.

For the tumor suppressive effects of c-Kit or c-Kit-EC after doxycycline injection, there were no totally inhibition of tumor growth, even though a significant difference could be obtained comparing to control group. There are at least two explanations for this phenomenon. First, we have performed intraperitoneally injection starting from 100mm<sup>3</sup> tumors, it was of doubt what was the percentage of cells that could obtain doxycycline. If doxycycline was not able to penetrate inside the tumors, it was possible that only cells on the tumor surface could obtain doxycycline, while the cells inside were absent from doxycycline and thus continued growing. Even if some cells obtained some doxycycline, whether the dose that it took was enough for c-Kit or c-Kit-EC induction was also of doubt. Second, the effect of one single injection of doxycycline didn't last long. Even we have injected everyday with doxycycline, if doxycycline was really unstable and the inducible effect was transient, we could not keep the tumor cells expression c-Kit or c-Kit-EC, which led to limited tumor suppressive effects.

### **Statistical analysis**

The data reported are the mean  $\pm$  S.E.M. of at least three independent determinations, each performed in triplicate. Statistical analysis was performed by the student t test.

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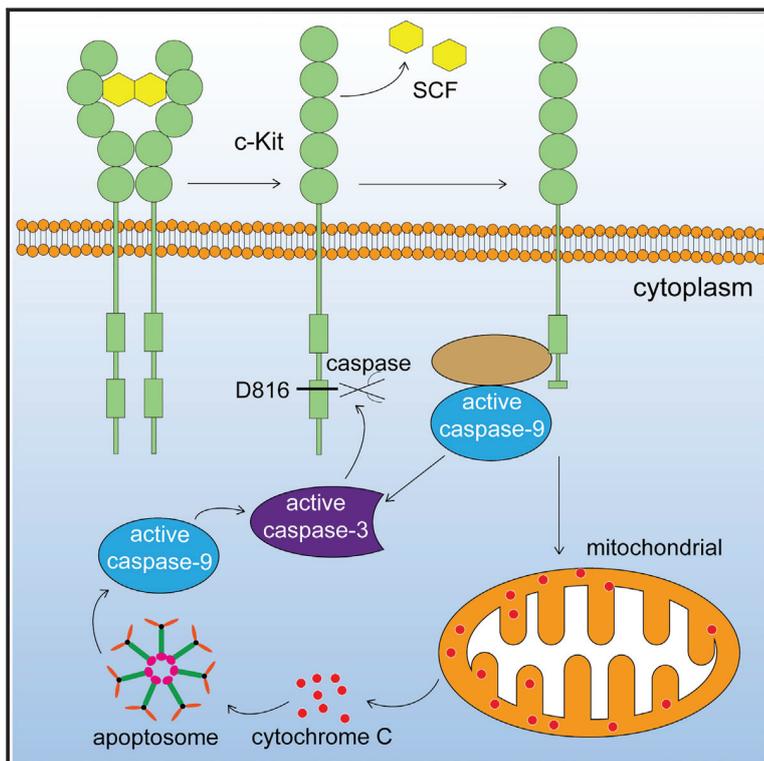
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# VII. Annexes

# Molecular Cell

## The Proto-oncogene c-Kit Inhibits Tumor Growth by Behaving as a Dependence Receptor

### Graphical Abstract



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### In Brief

c-Kit is seen as a proto-oncogene mutated or upregulated in cancer, leading to its constitutive kinase activation. We show that c-Kit triggers cancer cell death in settings of ligand absence and, thus, propose that c-Kit has two functions: pro-oncogenic activity via its kinase domain and tumor-suppressive activity via apoptosis induction.

### Highlights

- c-Kit, a well-known oncogene, actively triggers cell death in cancer cell lines
- c-Kit triggers cell death unless engaged by its ligand stem cell factor (SCF)
- The c-Kit death activity is due to intracellular cleavage of c-Kit in D816V
- c-Kit acts as a tumor suppressor via its dependence receptor activity

# The Proto-oncogene c-Kit Inhibits Tumor Growth by Behaving as a Dependence Receptor

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## SUMMARY

c-Kit is a classic proto-oncogene either mutated or upregulated in cancer cells, and this leads to its constitutive kinase activation and, thus, to uncontrolled proliferation. Although the pro-oncogenic role of c-Kit is of no doubt, some observations do not fit well with c-Kit solely as a tumor-promoting moiety. We show here that c-Kit actively triggers cell death in various cancer cell lines unless engaged by its ligand stem cell factor (SCF). This pro-death activity is enhanced when the kinase activation of c-Kit is silenced and is due to c-Kit intracellular cleavage by caspase-like protease at D816. Moreover, *in vivo*, overexpression of a c-Kit kinase-dead mutant inhibits tumor growth, and this intrinsic c-Kit tumor-suppressive activity is dependent on the D816 cleavage. Thus, c-Kit acts both as a proto-oncogene via its kinase activity and as a tumor suppressor via its dependence receptor activity.

## INTRODUCTION

Evasion of cell death is a hallmark of cancer (Hanahan and Weinberg, 2000), both for tumor transformation and resistance to anti-cancer agents (Hersey and Zhang, 2003). For this reason, cell death mechanisms and the re-engagement of cell death in cancer cells have been investigated in detail over the past 20 years. Although most of the literature and pharmaceutical efforts have been focused on both the extrinsic and intrinsic pathways for apoptosis (Meier and Vousden, 2007), over the past few years, an alternative pathway for cell death and its associated tumor-suppressive activity have emerged. This cell death pathway is induced by a functional family of membrane receptors dubbed dependence receptors. These receptors, despite their ability to transduce intracellular signals in the presence of their ligand, are also active in the absence of their ligand, and in these settings, they trigger apoptosis induction (Gibert and Mehlen,

2015; Paradisi and Mehlen, 2010). Expression of such dependence receptor renders cells dependent on the availability of the ligand for survival (Mehlen and Puisieux, 2006). To date, more than 20 receptors have been identified as dependence receptors (Gibert and Mehlen, 2015), sharing the ability to induce cell death in the absence of their respective ligand. This includes the netrin-1 receptors deleted in colorectal carcinoma (DCC) and UNC5H (Llambi et al., 2001; Mehlen et al., 1998), the Sonic Hedgehog receptors Patched and cell adhesion molecule-related/downregulated by oncogenes (CDON) (Delloye-Bourgeois et al., 2013; Thibert et al., 2003), and, more recently, Kremen1 (Causeret et al., 2016) and Notch3 (Lin et al., 2017). Because of this pro-death activity, most of them have been hypothesized to act as tumor suppressors, being downregulated in cancers (Bernet et al., 2007; Fearon et al., 1990; Shin et al., 2007; Thiebault et al., 2003). Along this line, silencing of the netrin-1 receptor DCC, the Sonic Hedgehog Homolog (SHH) receptor CDON, or the specific inactivation of the pro-death activity of DCC is associated with cancer progression in mice (Bernet et al., 2007; Castets et al., 2011; Delloye-Bourgeois et al., 2013; Krimpenfort et al., 2012). Of interest is that the receptors tyrosine kinase rearranged during transfection (RET), MET, and TrkC were also shown to act as dependence receptors (Bordeaux et al., 2000; Tauszig-Delamasure et al., 2007; Tulasne et al., 2004). We thus investigated whether another receptor tyrosine kinase, c-Kit, shown to be key for tumor progression, could also act as a dependence receptor.

The proto-oncogene c-Kit, also known as CD117, was initially identified as a viral oncogene in the Hardy-Zuckerman IV feline sarcoma virus (HZ4-FeSV) and is associated with its transforming activity (Besmer et al., 1986). It codes for a type III receptor tyrosine kinase (RTK), a class of membrane receptors playing a crucial role in cell proliferation, differentiation, and migration through different signaling pathways (Ali and Ali, 2007). Signaling of c-Kit is activated by the binding of its ligand, the stem cell factor (SCF) protein (Zsebo et al., 1990), causing c-Kit dimerization and activation by autophosphorylation and initiating a phosphorylation cascade resulting in the regulation of cell growth (Sattler and Salgia, 2004). Several studies have highlighted the major role of the SCF and c-Kit system in melanogenesis, gametogenesis, and hematopoiesis (Lennartsson et al., 2005) as well as in

brain angiogenesis (Sun et al., 2006). Moreover, dysregulation of c-Kit signaling or gain-of-function mutations are correlated with tumorigenesis, in particular in acute myeloid leukemia (AML), gastrointestinal stromal tumors (GISTs) and mastocytosis (Lennartsson et al., 2005). Gain-of-function somatic mutations trigger constitutive activation of c-Kit without SCF binding, leading to tumorigenesis. Moreover, expression of SCF and c-Kit has been reported in breast tumor cells, small-cell lung cancer cells, gynecological tumors, and malignant glioma (Lennartsson and Rönstrand, 2012). In neuroblastic tumors, such as neuroblastoma, the role of c-Kit function is still controversial. Although some reports showed no correlation between c-Kit and SCF and clinical characteristics, several groups have recently demonstrated that c-Kit expression is closely associated with differentiation and with a good prognosis (Krams et al., 2004). Indeed, the loss of expression of the c-Kit receptor may be related to neuroblastoma disease progression. This trait is not usually expected for an oncogene but is rather seen with dependence receptors, whose expression is often lost in cancer and leads to tumor progression and metastatic dissemination. We show here that the c-Kit receptor belongs to the dependence receptor family; thus, it is able to induce apoptotic cell death in the absence of its ligand SCF, which, in turn, is sufficient to inhibit apoptosis induction. Moreover, we show that c-Kit, despite its well-documented role as an oncogene, could have intrinsic tumor suppressor behavior via its pro-death activity.

## RESULTS

### c-Kit Intrinsically Triggers Apoptotic Cell Death

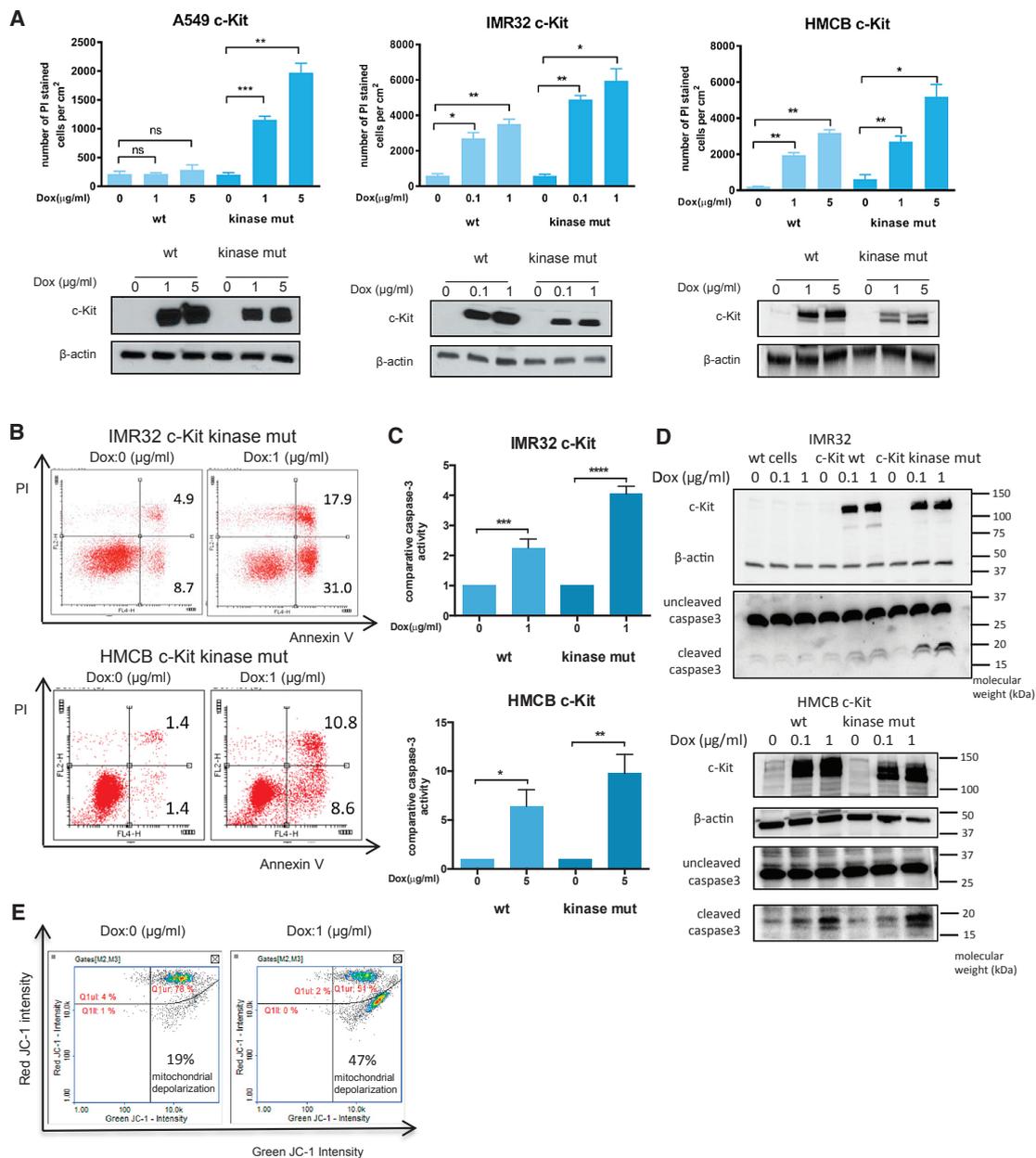
To study the effect of c-Kit on cell death, we generated several stable cell lines in which c-Kit expression is induced by adding doxycycline in the culture medium. To avoid positive signaling (i.e., kinase activation) that may induce survival signals, we generated kinase-mutated c-Kit-expressing cell lines using a c-Kit construct harboring the D792N mutation, which has been reported to be kinase activity-mutated (Roskoski, 2005). Inactivation of c-Kit kinase activity was verified by evaluating c-Kit auto-phosphorylation as well as ERK1/2 phosphorylation (Figure S1A). Moreover, kinase-inactivating mutation did not affect cellular membrane localization of c-Kit (Figure S1B). Interestingly, the inducible system used here, established from sleeping beauty vectors, allowed c-Kit expression at levels comparable with endogenous levels detected in M2G2 and zeroderma pigmentosum, complementation group C (XPC) melanoma cell lines (Figure S1C). Forced expression of wild-type (WT) or mutant c-Kit following doxycycline treatment induced a dose-dependent increase of propidium iodide (PI)-positive cells, compared with untreated cells, in neuroblastoma (IMR32) or melanoma (human melanoma cell bowes [HMCB]) cell lines (Figures 1A, S1D, and S1E). In A549 cell line, overexpression of WT c-Kit was not sufficient to induce cell death, whereas the kinase-dead c-Kit massively enhanced cell death. We investigated whether the cell death induced by c-Kit overexpression was apoptosis by assessing the Annexin V staining rate in IMR32 and HMCB cell lines upon treatment with doxycycline. Overexpression of kinase-mutated c-Kit induced an increase in the Annexin V-positive population compared with untreated cells (Figure 1B).

The implication of apoptosis was further demonstrated by measuring caspase activity. As shown in Figure 1C, overexpression of c-Kit was able to strongly induce caspase activity compared with untreated IMR32 and HMCB cells, especially kinase-mutated c-Kit. Moreover, c-Kit overexpression was also sufficient to induce caspase-3 cleavage (Figure 1D), a specific hallmark of the apoptotic process. Because apoptosis induction is often associated with mitochondrial outer membrane permeabilization (MOMP), a critical event governing the release of pro-apoptotic molecules (Kalkavan and Green, 2018; Li et al., 1997), we showed that c-Kit forced expression in HMCB cells induced MOMP (Figure 1E). Finally, the implication of caspase in cell death induced by c-Kit was confirmed by analyzing cell death upon treatment with the pan-caspase inhibitor Q-VD-oxo-pentanoic acid hydrate (Q-VD-OPh). Caspase inhibition completely suppressed DNA fragmentation induced by kinase-mutated c-Kit overexpression (Figure 2A). Additionally, Q-VD-OPh inhibited the increase of the Annexin V-positive population induced by kinase-mutated c-Kit overexpression in HMCB cells (Figure S1F). In a similar way, the use of a caspase-3 inhibitor prevented PI positivity induced by WT or kinase mutant c-Kit overexpressed in A549 and IMR32 cells (Figure 2B). To further analyze which caspases were involved in c-Kit-induced cell death, we generated stable HMCB cells inducible for c-Kit and knocked down for caspase-3 and caspase-9 using the CRISPR/Cas9 strategy. Knocking down caspase-3 or caspase-9 was sufficient to inhibit DNA fragmentation observed upon c-Kit induction in HMCB cells (Figure 2C). Moreover, we observed a similar inhibitory effect on the Annexin V-positive population induced by c-Kit overexpression in IMR32 cells (Figures S1G and S1H). Collectively, these data strongly suggest that c-Kit actively triggers cell death and that this death activity is, at least in part, masked by c-Kit kinase activity.

### c-Kit-Induced Apoptosis Is Inhibited by Its Ligand SCF and Is Mediated by Intracellular Caspase-like Cleavage Driving a Caspase Activation Amplification Loop

We then assessed whether, as expected for a dependence receptor, the ligand of c-Kit could inhibit cell death induction (Goldschneider and Mehlen, 2010). As shown in Figure 3A, although, in the A549 cell line, the induction of kinase-mutated c-Kit strongly increased the number of PI-positive cells, treatment with SCF clearly inhibited cell death induced by doxycycline treatment. Similarly, treatment of IMR32 cells with SCF was sufficient to inhibit caspase-3 activation and cleavage induced by c-Kit overexpression (Figures 3B and S2A) as well as to decrease the percentage of Annexin V-positive cells (Figure S2B). These results show that the ligand SCF is able to revert apoptosis induced by c-Kit.

Caspases appear to be key components in mediating the death of most dependence receptors, and most of them are cleaved by caspase in their intracellular domain as a prerequisite for cell death engagement. To assay whether c-Kit could also be a substrate for caspases, we translated *in vitro* the intracellular region of c-Kit, and we incubated this domain with purified active caspase. As shown in Figure 3C, this intracellular domain was cleaved *in vitro* by active caspase, generating cleavage products that migrated at molecular masses of 35 and 18 kDa. To identify the putative caspase cleavage site, we systematically mutated



### Figure 1. c-Kit Induces Apoptosis In Vitro

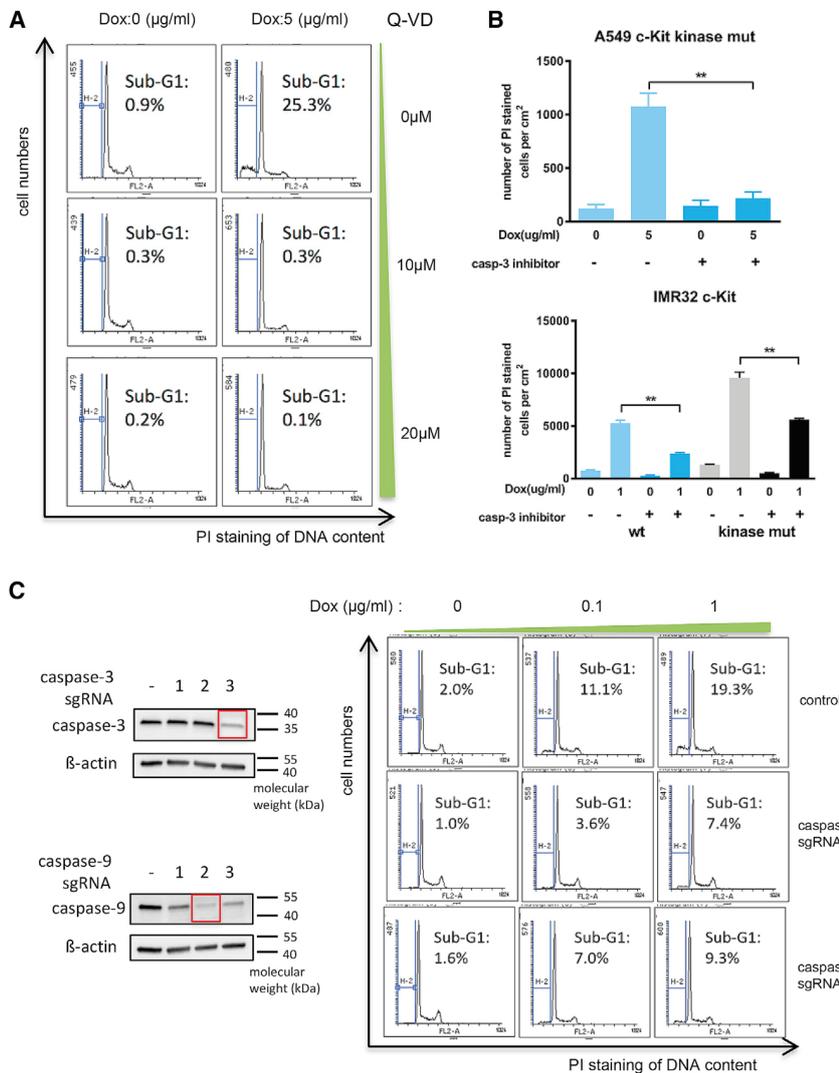
(A) c-Kit overexpression triggers cell death. A549, IMR32, and HMCB stable cell lines inducible for wild-type (WT) or kinase-mutated (kinase-mut) c-Kit were treated with the indicated amount of doxycycline (Dox) in a 96-well plate (A549 and IMR32) or 6-well plate (HMCB). Cell death was analyzed by propidium iodide (PI) staining 36 hr (IMR32 and HMCB) or 48 hr (A549) after Dox treatment using the Incucyte Zoom instrument. Expression of c-Kit proteins upon Dox treatment was confirmed by western blot (bottom).

(B) c-Kit-induced cell death is associated with phosphatidylserine flipping to the extracellular surface of the cell, a specific hallmark of apoptotic cell death (Vermes et al., 1995). IMR32 and HMCB stable cell lines were treated with Dox, and the apoptosis rate was quantified by AnnexinV and PI staining through flow cytometry. The top right quadrant represents double-positive cells (late apoptosis), whereas cells positive only for Annexin V (early apoptosis) are present in the bottom right quadrant. The numbers in the quadrants correspond to the percentage of cells.

(C and D) c-Kit is able to activate caspase-3. IMR32 and HMCB stable cell lines were treated with Dox to induce c-Kit, and active caspase-3 was quantified by fluorometric detection kit (C). Caspase-3 activity was normalized to total protein amount and indexed to untreated cells. Caspase-3 activation was also evaluated by western blot (D), using a caspase-3 antibody able to recognize uncleaved (inactive) or cleaved (active) protein.

(E) c-Kit promotes mitochondrial outer membrane permeabilization (MOMP). The HMCB stable cell line was treated with Dox, and the mitochondrial depolarization rate was quantified by JC-1 dye staining. The low right quadrant represents mitochondrial depolarized cells. The numbers in the quadrants corresponds to the percentage of total cells.

Data are representative of at least three replicates as mean  $\pm$  SEM. ns, non-significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 2. c-Kit Induces Caspase-Dependent Apoptosis**

(A and B) c-Kit-induced apoptosis requires caspase-3 activation. HMCB (A) and A549 and IMR32 (B) stable cell lines were treated with Dox and the pan-caspase inhibitor Q-VD-Oph hydrate (Q-VD) or the specific caspase-3 inhibitor, and cell death was evaluated by DNA fragmentation assay (A) or by PI staining (B).

(C) Caspase-3 and caspase-9 deletion protects cells from c-Kit-induced apoptosis. Caspase-3 and caspase-9 were knocked down separately in the HMCB stable cell line using the CRISPR/Cas9 technique, assessed by three caspase-3 or caspase-9 guide RNAs. The knockdown efficiency of these guide RNAs was evaluated by western blot (left). Small guide RNA (sgRNA)-3 for caspase-3 and sgRNA-2 for caspase-9, showing better knockdown efficiency, were chosen for further experiments. The HMCB stable cell line was then treated with Dox, and cell death was evaluated by DNA fragmentation assay (right).

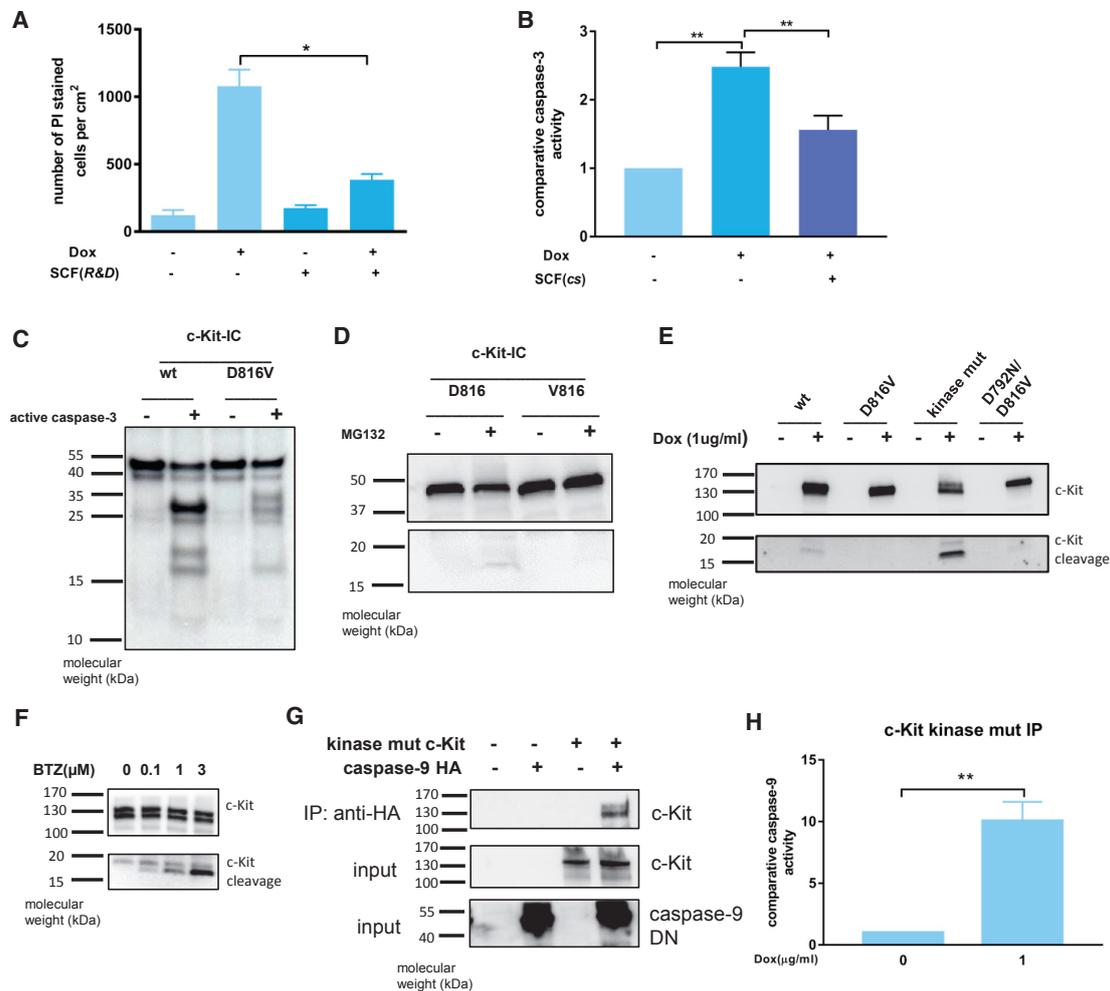
Data are representative of at least three replicates as mean  $\pm$  SEM. \*\* $p < 0.01$ .

inhibitor of apoptosis protein (cIAP) and X-linked inhibitor of apoptosis protein (XIAP) inhibitor (Li et al., 2011) or PAC1, a potent direct procaspase-3 activator (Putt et al., 2006; Figure S2E). Thus, together, these data support the view that c-Kit is cleaved by caspase-like protease *in vitro* and in cell culture at Asp-816. Of key interest is that the D816V c-Kit mutation has been extensively reported in the literature as a mutation associated with tumor resistance to Gleevec (Frost et al., 2002).

Another trait shared by several dependence receptors is their ability, when cleaved, to recruit a protein complex encompassing and activating caspase-9 (Delloye-Bourgeois et al., 2013; Forcet et al., 2001; Lin et al., 2017; Mille et al., 2009). Because caspase-9 has been shown to be required for c-Kit-induced apoptosis, we investigated whether c-Kit could interact with and activate caspase-9. We thus performed a co-immunoprecipitation assay between c-Kit and caspase-9. As shown in Figure 3G, caspase-9 efficiently pulls down kinase-mutated c-Kit. To investigate further whether c-Kit, when expressed upon doxycycline treatment, could not only interact with but could also activate caspase-9, we performed c-Kit pull-down and assessed the caspase activity contained in the pull-down. As shown in Figure 3H, c-Kit pull-down retains large caspase-9 activity.

To explore the role of caspase cleavage in c-Kit-induced apoptosis, we next generated stable cell lines inducible for double-mutated c-Kit (D792N/D816V), thus lacking kinase activity and the possibility to be cleaved by caspases. Although the double mutation did not affect cellular membrane localization of c-Kit (Figure S1B), mutation of the putative caspase cleavage site

aspartic acid residues that could match the cleavage products. Of interest, the mutation in the intracellular region of Asp-816 to valine (D816V) abolished cleavage and the generation of the fragments at 35 and 18 kDa (Figure 3C). Moreover, we confirmed c-Kit cleavage by caspases in cells by generating IMR32 and A549 cell lines stably expressing the intracellular region of c-Kit (Figures 3D and S2C). In presence of the proteasome inhibitor MG132, used here to increase the stability of the cleaved fragment, a fragment migrating at a molecular mass of 18 kDa appeared in stable cells expressing WT c-Kit, whereas this band was not detected when we generated the D816V mutated construct. A similar 18-kDa fragment was observed when stable cells expressing full-length c-Kit were treated with bortezomib, another proteasome inhibitor (Adams et al., 1999; Figure 3E). In addition, we confirmed the cleavage of endogenous c-Kit, treating melanoma M2Ge cells with bortezomib or MG132 proteasome inhibitors (Figures 3F and S2D). Moreover, c-Kit caspase cleavage was enhanced by treating M2Ge cells with bortezomib and caspase activators such as BV6, a dual cellular



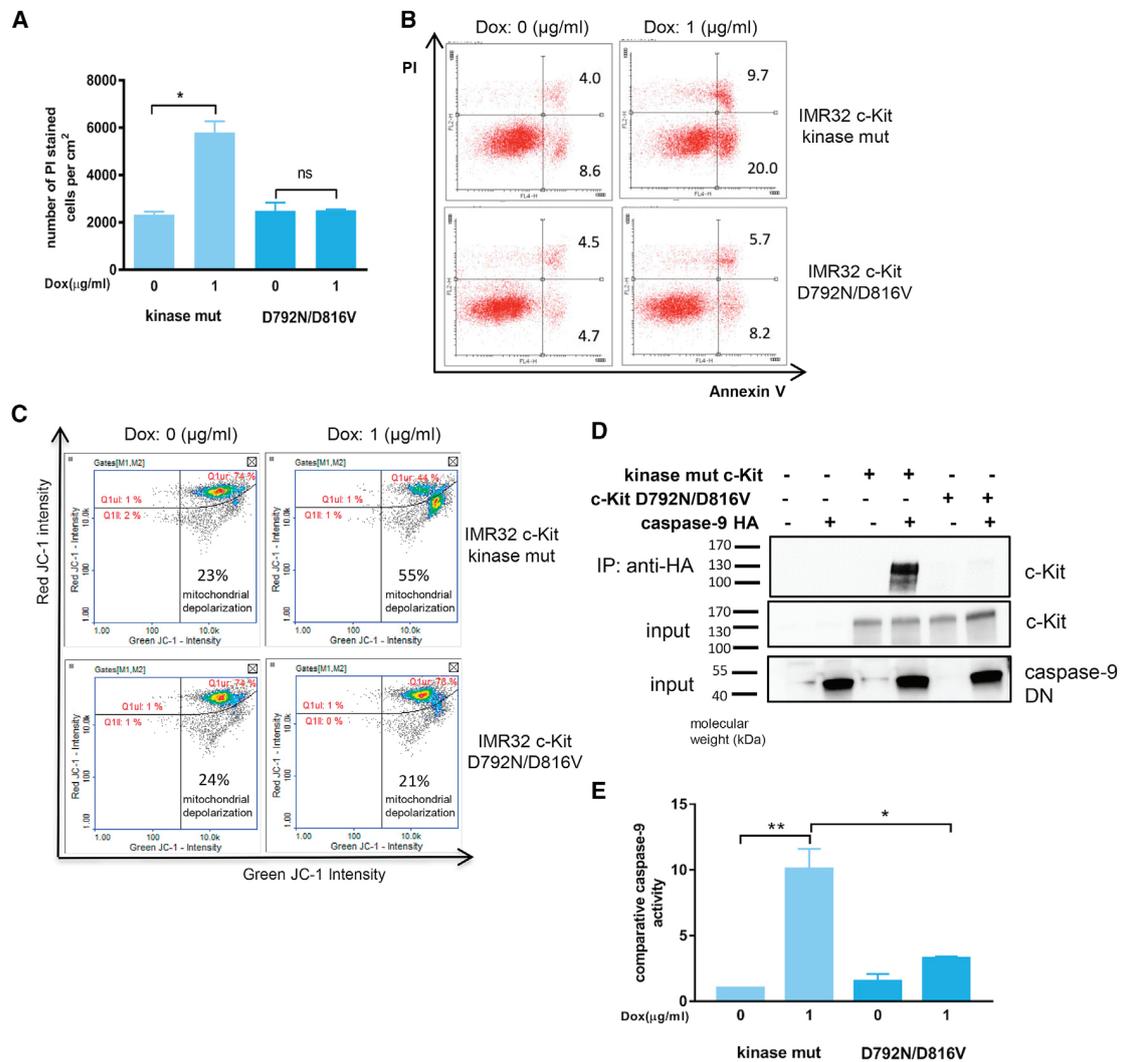
**Figure 3. c-Kit-Induced Apoptosis Is Inhibited by SCF and Is Mediated by Intracellular Caspase-like Cleavage Driving a Caspase Activation Amplification Loop**

(A and B) The ligand SCF inhibits c-Kit-induced cell death. A549 and IMR32 stable cell lines inducible for kinase-mut c-Kit were treated with Dox and recombinant SCF proteins derived from *E. coli* (R&D Systems, 1 μg/mL) or from 293T cells (Cell Signaling Technology, 25 ng/mL). Apoptotic cell death was then evaluated in the A549 stable cell line by PI staining 48 hr after treatment and quantified with the Incucyte Zoom instrument (A) and in the IMR32 stable cell line by caspase-3 activation and quantified with a fluorometric detection kit (B).

(C–F) c-Kit is cleaved by caspase-3. The *in vitro*-translated intracellular region of WT or caspase cleavage site-mutated (D816V) c-Kit was incubated with 0.3 μM of purified active caspase-3, and digested bands were analyzed by autoradiography (C). Although WT c-Kit cleavage revealed the presence of two major bands at 31 kDa (corresponding to a c-Kit fragment from the transmembrane domain to D816) and 18 kDa (c-Kit fragment to D816 to stop codon), these bands disappeared when using the D816V mutant. c-Kit cleavage was then confirmed in IMR32 cells transfected with the kinase-mut (D816) or kinase- and caspase-mutated (V816) intracellular region of c-Kit (D). After treatment for 2 hr with the proteasome inhibitor MG132, western blot analysis using a C-terminal hemagglutinin (HA) tag revealed the presence of a band at 18 kDa that disappeared in the V816 construct. Cleavage at the D816 site of full-length c-Kit was also confirmed by using IMR32 stable inducible cell lines (E) in which c-Kit or its mutants was induced by Dox. After a 2-hr treatment with the proteasome inhibitor bortezomib, western blot analysis using an antibody targeting the C-terminal region of c-Kit revealed the presence of a band at 18 kDa in WT and kinase-mut (D792N) c-Kit but not in c-Kit stable cells mutated at the D816 site. The western blot presented here is representative of three independent experiments. In addition, endogenous c-Kit could also be cleaved (F). In the M2Ge melanoma cell line, an 18-kDa band was detected by c-Kit antibody targeting the C-terminal region in the presence of bortezomib.

(G and H) c-Kit interacts with and activates caspase-9. 293T cells were transfected with kinase-mut c-Kit and caspase-9-HA plasmids. After lysis, caspase-9 was pulled down by HA antibody, and c-Kit was detected in the immunoprecipitated fraction using α-c-Kit antibody (G). IMR32 stable cells, inducible for kinase-mut c-Kit, were treated with Dox for 24 hr, and, after lysis, kinase-mut c-Kit was pulled down by FLAG antibody. Active caspase-9 that bound to pulled down c-Kit was quantified with a caspase-9 activity detection kit (H).

Data are representative of at least three replicates as mean ± SEM. \*p < 0.05, \*\*p < 0.01.



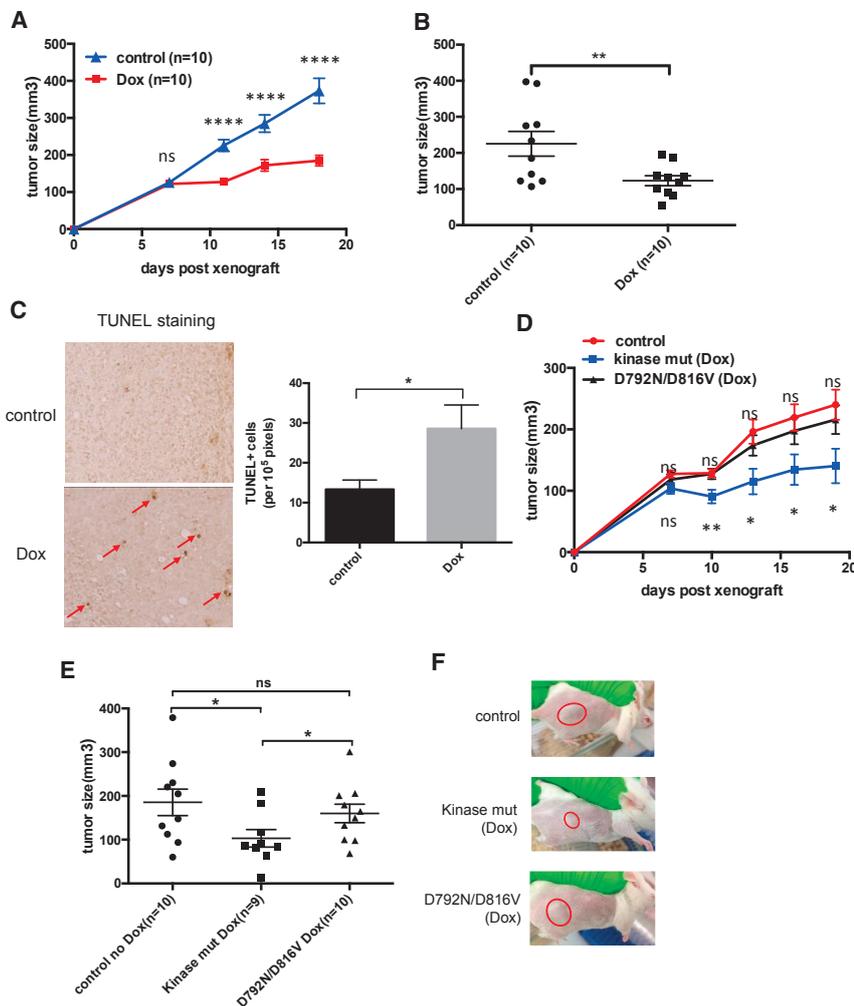
**Figure 4. c-Kit-Induced Apoptotic Cell Death Requires c-Kit Caspase-3 Cleavage**

(A–C) c-Kit caspase cleavage is required for cell death induction. The IMR32 stable cell line inducible for kinase-mut or kinase- and caspase-mutated (D792N/D816V) c-Kit was treated with the indicated Dox concentrations. Apoptotic cell death was then evaluated by PI staining 36 hr after Dox treatment in a 96-well plate (A) and by AnnexinV and PI double staining analyzed by flow cytometry (B). In addition, D816V c-Kit was not able to induce MOMP, promoted by kinase-mut c-Kit, as indicated by mitochondrial depolarization quantified by JC-1 dye staining (C).

(D and E) The D816V mutation impairs formation of the caspase-activating complex between c-Kit and caspase-9. 293T cells were transfected with kinase- or double-mutated c-Kit and caspase-9-HA plasmids (D). After lysis, caspase-9 was pulled down by HA antibody, and c-Kit was detected in the immunoprecipitated fraction using  $\alpha$ -c-Kit antibody. Moreover, IMR32 stable cells, inducible for kinase- or double-mutated c-Kit, were treated with Dox for 24 hr, and, after lysis, kinase-mut c-Kit was pulled down by FLAG antibody (E). Active caspase-9 was then quantified in the pull-down fraction with a caspase-9 activity detection kit. Data are representative of at least three independent experiments as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ .

dramatically altered the pro-apoptotic activity of kinase-mutated c-Kit. Indeed, in the IMR32 and A549 cell lines, overexpression of double-mutated c-Kit was not able to increase PI positivity (Figures 4A and S2F) compared with kinase-mutated c-Kit. Moreover, mutation of the putative caspase site at D816 inhibited the increase in the Annexin V-positive population observed after induction of kinase-mutated c-Kit (Figure 4B) as well as caspase-3 cleavage (Figure S2G). In a similar way, the D816V mutation affected the ability of c-Kit to promote MOMP (Figure 4C). We then investigated the relationship between caspase

cleavage of c-Kit and caspase-9 recruitment and activation. Interestingly, caspase-mutated c-Kit was not able to interact with caspase-9 (Figure 4D). Moreover, when performing a pull-down of c-Kit after doxycycline treatment, the caspase-9 activity included in the pull-down was dramatically reduced when double-mutated c-Kit was pulled down instead of kinase-mutated c-Kit (Figure 4E). Collectively, these results show that c-Kit shares the general traits of a dependence receptor, triggering cell death via a mechanism requiring cleavage of its intracellular domain at D816, a key amino acid involved in tumor resistance.



**Figure 5. c-Kit Overexpression Induces Tumor Regression *In Vivo***

(A–C) SCID (severe combined immunodeficiency) mice engrafted with the HMBC kinase-mut c-Kit stable cell line were intraperitoneally treated every day with Dox (50 mg/kg diluted in H<sub>2</sub>O) or vehicle. Tumor sizes were measured on the indicated days after xenograft (A) and after the mice were sacrificed (B). Apoptotic cells in tumor sections revealed by TUNEL staining are indicated by red arrows (representative images), and TUNEL-positive cells were quantitated (C).

(D–F) The HMBC stable cell line inducible for kinase-mut or kinase- and caspase-mutated (D792N/D816V) c-Kit was engrafted in SCID mice. Mice were intraperitoneally treated every day with Dox (50 mg/kg diluted in H<sub>2</sub>O) or vector. Tumor sizes were measured on the indicated days after xenograft (D) and after the mice were sacrificed (E). Photos of representative mice are shown right before sacrificing the mice (F). Tumors are indicated by red circles.

Data are representative as mean ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001.

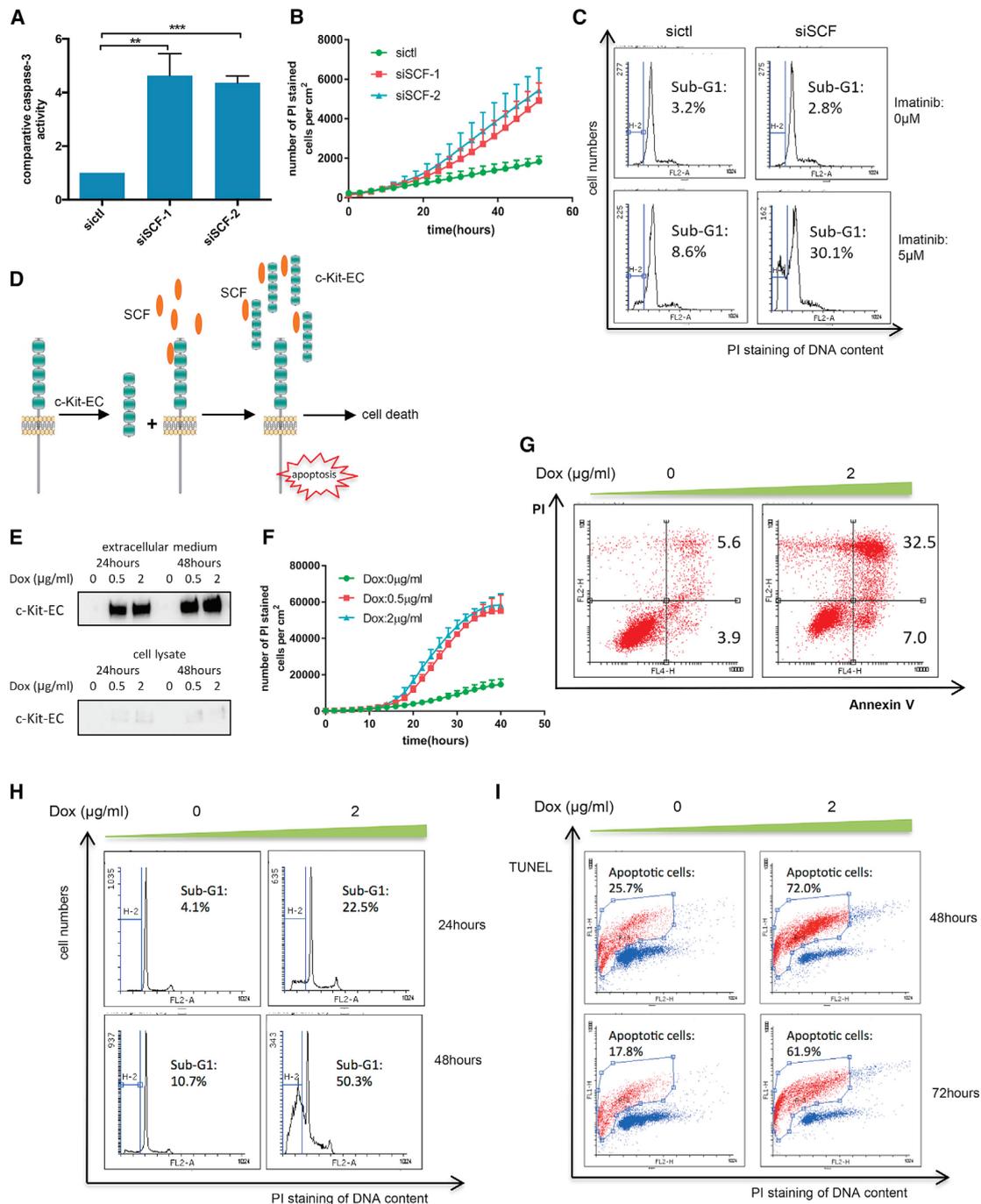
### c-Kit Acts as a Tumor Suppressor

Most of the described dependence receptors act as tumor suppressors. We thus hypothesized that c-Kit, known as a classic proto-oncogene because of its kinase activity, could actually intrinsically hold tumor-suppressive activity that is somehow masked by the c-Kit kinase activity. To show this, HMBC cells, inducible for kinase-mutated c-Kit, were engrafted in severe combined immunodeficiency (SCID) mice, and animals with tumors were treated daily by intraperitoneal (i.p.) injections of doxycycline (or vehicle) to induce c-Kit expression in engrafted tumors (Figures S3A and S3B). Although vehicle injection had no effect on tumor growth, expression of kinase-dead c-Kit induced strong and prolonged tumor growth inhibition (Figure 5A). Moreover, the final tumor size was significantly lower in the doxycycline-treated tumor group compared with the control-treated group (Figure 5B). Quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells on tumor sections from recovered tumors also indicated that kinase-mutated c-Kit was able to trigger more cancer cell death *in vivo* (Figure 5C).

To more formally demonstrate the role of c-Kit pro-apoptotic activity in this tumor-suppressive effect, we engrafted the HMBC cell line, inducible for kinase-dead c-Kit mutated in its caspase cleavage site (D792N/D816V). Mutation of the caspase site was sufficient to prevent the tumor suppressor activity of c-Kit because expression of kinase- and caspase-mutated c-Kit had no more tumor growth inhibiting effect (Figure 5D), despite a similar expression of c-Kit and c-Kit D792N/D816V in both stable cell lines (Figure S3C). In addition, there was no difference between final sizes of tumors obtained in double-mutant c-Kit cells and injected with vehicle or doxycycline, whereas tumors obtained from kinase-mutated c-Kit, harboring a wild-type caspase site, were significantly smaller (Figures 5E and 5F).

### SCF Interference Triggers Apoptosis and Limits Tumor Growth

Following this hypothesis, blocking interaction of SCF with c-Kit should both inhibit the kinase activity and unleash the killing activity. We thus investigated whether, in cancer cell settings, the SCF autocrine production reported extensively (Lennartsson and Rönnstrand, 2012; Théou-Anton et al., 2006) could not only activate the c-Kit kinase activity but also block the death activity of endogenous c-Kit. To demonstrate that endogenous c-Kit was able to trigger apoptosis in the absence of SCF, we silenced the ligand using the RNAi strategy in various cancer cells. A549 cells transfected with small interfering RNAs (siRNAs) specific for SCF showed a strong increase in caspase-3 activity compared with cells transfected with a control, non-specific



**Figure 6. SCF Silencing or Interference Triggers Apoptosis**

(A and B) SCF silencing induces apoptotic cell death. A549 cells were transfected either with sic or with two specific siRNAs targeting SCF (siSCF-1 and siSCF-2). Apoptosis was evaluated by caspase-3 activity, measured 24 hr after siRNA transfection with a fluorometric detection kit (A) and by PI staining quantified with the Incucyte Zoom instrument (B). Data are representative of at least three independent experiments.

(C) Combining SCF silencing and imatinib treatment triggers apoptosis in resistant cells. M2Ge melanoma cells were transfected either with a scramble siRNA (sic1) or with a specific siRNA targeting SCF (siSCF). 24 hr after SCF silencing, cells were treated with imatinib for a further 24 hr, and the apoptosis rate was measured by DNA fragmentation assay through flow cytometry.

(D) Schematic representation of the mode of action of SCF interference by the extracellular region of c-Kit. The binding between SCF and c-Kit could be inhibited by the extracellular domain of c-Kit (c-Kit-EC), which interacts with SCF, acting as a trap able to interfere with SCF and c-Kit binding; thus, it could trigger apoptotic cell death induced by the unbound c-Kit receptor.

(legend continued on next page)

siRNA (Figures 6A and S4A). We found a similar effect by transfecting the HCT116 cell line with SCF siRNAs (Figure S4B) expressing very high levels of the ligand. Moreover, SCF silencing enhanced the number of PI-positive cells in transfected A549 cells (Figure 6B). In A549 and HCT116 cells, the endogenous level of c-Kit is relatively low, supporting the view that, in these cell lines, there is no spontaneous auto-activation of the kinase activity of c-Kit that may interfere with the pro-apoptotic activity of c-Kit. Of interest, in melanoma M2Ge cells, expressing high levels of the receptor and, thus, showing receptor auto-activation (Figure S1C), silencing of SCF failed to affect cell viability (Figure 6C). However, when, in these M2Ge cells, we inhibited kinase activity using imatinib treatment, we observed massive enhancement of cell death induction upon SCF silencing (Figure 6C).

Next we investigated a way to interfere with the binding between c-Kit and SCF in a more therapeutically relevant manner than RNAi. For this purpose, we generated a stable HCT116 cell line, inducible for the expression of the extracellular domain of c-Kit (c-Kit-EC; Figure 6D), secreted into the extracellular milieu (Figure 6E). This recombinant protein has been shown to interact with SCF (Philo et al., 1996), probably acting as a trap able to interfere with the binding between SCF and the membrane-bound c-Kit (Lev et al., 1992). Overexpression of c-Kit-EC upon treatment with doxycycline triggered apoptotic cell death, as shown by induction of a strong increase in the number of PI-positive cells (Figure 6F), the Annexin V-positive population (Figure 6G), DNA fragmentation (Figure 6H), and TUNEL-positive cells (Figure 6I). Moreover, conditioned medium (CM) obtained upon induction of the c-Kit-EC-stable HCT116 cell line was able to induce an increase in the Annexin V-positive population (Figure S4C) and DNA fragmentation (Figure S4D) in parental HCT116 cells compared with control CM.

To demonstrate that cell death triggered by interference with SCF and c-Kit binding was due to the pro-apoptotic activity of c-Kit rather than an indirect effect of switching off the survival kinase-dependent signal, we first used a c-Kit RNAi strategy. If c-Kit acted only via its kinase activity, then silencing of c-Kit should mimic the effect of the induction of c-Kit-EC. However, if c-Kit really acted as a dependence receptor, then silencing of c-Kit should somehow revert the effect of c-Kit-EC. As shown in Figures 7A and S4E, transfection of stable HCT116 cells inducible for c-Kit-EC with c-Kit-specific siRNA is sufficient to revert the increase in the Annexin V-positive population observed upon induction of c-Kit-EC. To confirm the pivotal role of the endogenous c-Kit receptor to induce apoptosis, we generated, using the CRISPR/Cas9 strategy, two stable HCT116 cell lines knocked out for c-Kit, obtained by two different c-Kit-specific guide RNAs (Figure S4F). Although the parental HCT116 cell line showed an increase in the Annexin V-positive population (Figure 7B) or DNA fragmentation rate (Figure 7C) following treat-

ment with CM obtained upon c-Kit-EC induction, treatment of c-Kit knockout cell lines had no effect on apoptosis induction (Figures 7B and 7C). These results confirmed that cell death induction by interference with SCF and c-Kit binding was triggered by c-Kit pro-apoptotic activity.

These *in vitro* data support the view that interfering with the SCF and c-Kit interaction could be of interest in a therapeutic setting. A stable HCT116 cell line inducible for c-Kit-EC was thus engrafted in SCID mice, and animals with tumors were treated daily with i.p. injections of doxycycline to induce c-Kit-EC expression (Figure S4G) and interfere with SCF and c-Kit binding as a means to activate the pro-apoptotic activity of endogenous c-Kit. Doxycycline treatment significantly slowed down tumor growth (Figure 7D) and decreased final tumor sizes (Figures 7E and 7F) compared with vehicle-treated animals. Taken together, these data support the hypothesis that targeting the SCF and c-Kit interaction to stimulate c-Kit pro-apoptotic activity could be investigated further as a complementary therapeutic strategy to c-Kit kinase inhibitors.

## DISCUSSION

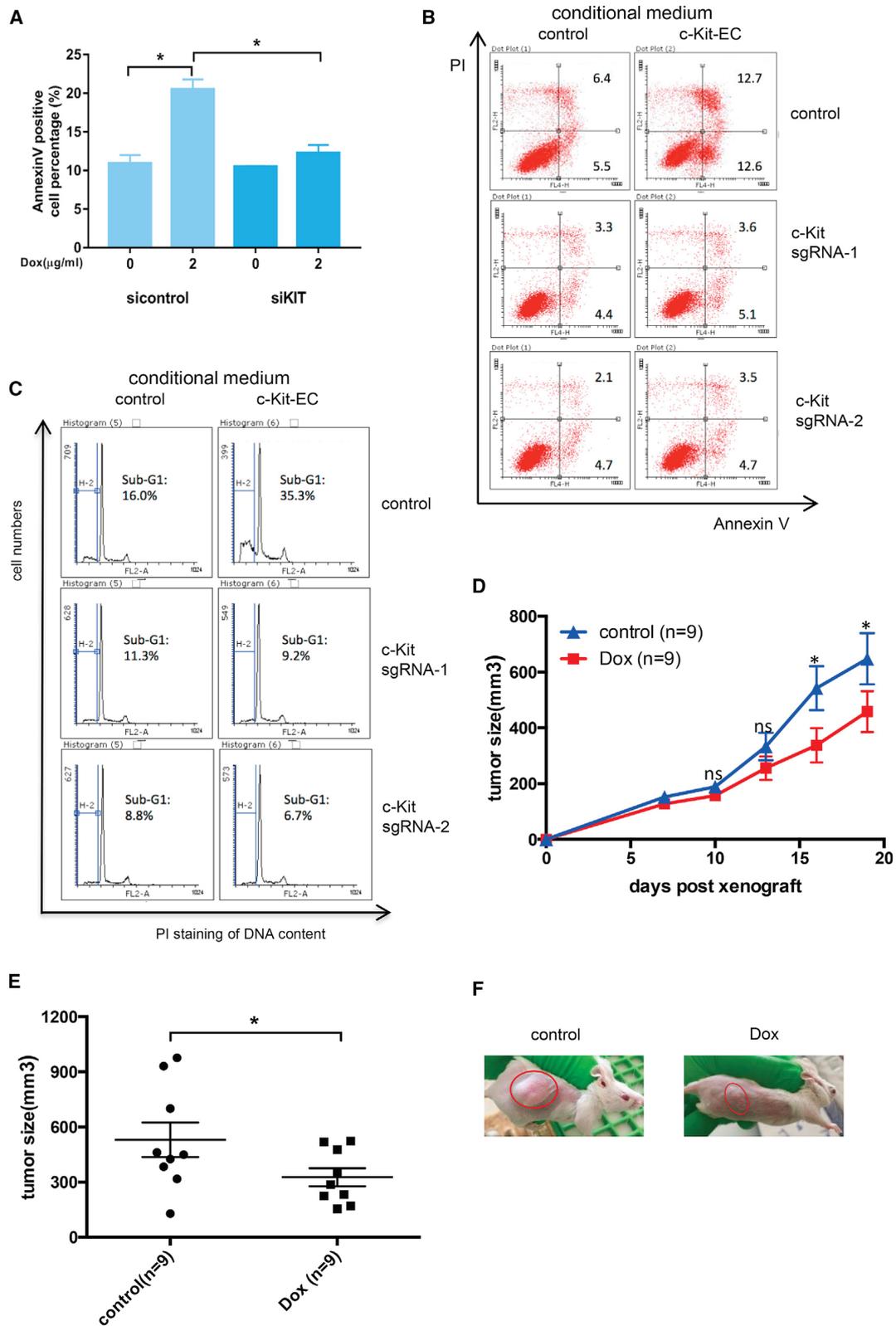
The proto-oncogene c-Kit has been classically considered only as a receptor tyrosine kinase, and most of the studies of c-Kit focused on its kinase activity. Hence, therapeutic strategies targeting c-Kit have been centered on inhibition of its kinase activity, as exemplified by the development of imatinib (Gleevec), blocking ATP binding to the c-Kit active site (Buchdunger et al., 2002). Although imatinib has been proven to be effective in several cancers, such as c-Kit-positive GISTs, 10% of patients show primary resistance, and a vast majority of patients eventually develop resistance to the drug because of mutations in KIT that render GIST cells imatinib-resistant and allow c-Kit signaling to remain constitutively active (Corless et al., 2011). We propose here that, rather than inhibiting c-Kit kinase activity, an alternative therapeutic strategy could be to target the interaction between SCF and c-Kit to both inhibit the kinase activity and to induce c-Kit-induced cell death, at least in patients showing autocrine and/or paracrine production of c-Kit ligand.

Several hints have led us to formulate this hypothesis. Indeed, it has been shown that removal of SCF or addition of a monoclonal c-Kit antibody triggers apoptosis in murine melanocyte precursors (Ito et al., 1999). Moreover, SCF promotes mast cell survival by suppressing apoptosis (Iemura et al., 1994). In addition, c-Kit expression is lost in neuroblastoma (Krams et al., 2004), a disease that originates from migrating neural crest cells from which melanocyte precursors also arise. Along this line, the loss of expression of the c-Kit receptor may be related to neuroblastoma disease progression (Shimada et al., 2008). Furthermore, c-Kit downregulation has been described in invasive melanoma, thyroid carcinoma, and breast cancer (Natali et al.,

(E) c-Kit-EC is secreted in the extracellular milieu. HCT116 stable cells inducible for c-Kit-EC were treated with the indicated concentrations of Dox, and the presence of the recombinant protein was evaluated by western blot in the extracellular medium (top) or in cell lysate (bottom).

(F–I) Overexpression of c-Kit-EC triggers apoptotic cell death. The HCT116 stable cell line inducible for c-Kit-EC was treated with the indicated Dox concentrations. Induction of cell death was then evaluated by PI staining measured by the Incucyte Zoom device (F), by AnnexinV and PI double staining by flow cytometry (G), and by DNA fragmentation (H) and TUNEL (I) assays.

Data are representative of at least three independent experiments as mean  $\pm$  SEM. \*\*p < 0.01, \*\*\*p < 0.001.



(legend on next page)

1992a, 1992b, 1995). These findings did not fit easily with a typical profile of a proto-oncogene. This matches much better with c-Kit being a dependence receptor. The pro-death activity of dependence receptors has been shown to confer tumor suppressor activity to these receptors, and, accordingly, they are often lost or downregulated in several human cancers, including colorectal, breast, ovary, uterus, stomach, lung, or kidney cancer (Bernet et al., 2007; Genevois et al., 2013; Mehlen and Fearon, 2004; Thiebaut et al., 2003). Of interest is that although, in a large fraction of cancers, dependence receptors are downregulated, in other cancers, a similar selective advantage is gain of the ligand (Bouzas-Rodriguez et al., 2010; Delloye-Bourgeois et al., 2009, 2013; Fitamant et al., 2008; Paradisi et al., 2008, 2013). In this setting, autocrine production of the ligand of a dependence receptor represents a survival advantage, inhibiting tumor cell death and, accordingly, promoting tumor progression and metastasis (Mehlen et al., 2011). Targeting ligand and dependence receptor interaction in cancer with upregulation of dependence receptor (DR) ligand is currently assessed in an early clinical trial for the pair netrin-1 and UNC5B (<https://clinicaltrials.gov/ct2/show/NCT02977195>). Of interest is that although c-Kit amplification and constitutive kinase activation is one of the major modes of action for promoting tumor progression, autocrine production of SCF has also been extensively described (Lennartsson and Rönstrand, 2012; Théou-Anton et al., 2006). Even though, so far, this gain of SCF is mainly seen as a mechanism to promote c-Kit kinase activation, our data suggest that it may also block c-Kit-induced apoptosis.

Including c-Kit in the functional family of dependence receptors may have important therapeutic consequences. Indeed, in cancers showing SCF expression, inhibiting the kinase activity may not be sufficient to efficiently trigger cell death of tumor cells. Thus, a co-treatment based on both kinase inhibition and stimulation of the pro-apoptotic activity of these tyrosine kinase dependence receptors could appear as a more attractive and efficient therapeutic strategy that may bypass some of the currently observed tumor resistance. We have started here to validate this hypothesis, using the c-Kit extracellular domain to induce cell death and tumor growth inhibition in SCF-expressing HCT116 cancer cells. Of interest, alongside our hypothesis is the observation that the c-Kit caspase cleavage mutation appears to be detected in human tumors upon resistance to Gleevec. Even though, so far, the mechanism of action for this mutation is generally considered as the change in accessibility of Gleevec to c-Kit (Gajiwala et al., 2009), further studies should investigate whether the D816V mutation is also selected because it prevents

c-Kit to eliminate cancer cells. Further work will unravel whether combining a kinase inhibitor and an SCF and c-Kit-blocking biologic agent could be more efficacious and associated with less resistance.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <https://doi.org/10.1016/j.molcel.2018.08.040>.

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## AUTHOR CONTRIBUTIONS

H.W. performed and analyzed most of the experiments. A.B., L.M., S.T.-D., Y.S., and D.G. contributed to some important experiments. A.P. and P.M. supervised the work and wrote the manuscript.

### Figure 7. SCF Interference Triggers c-Kit-Dependent Apoptosis and Limits Tumor Growth *In Vivo*

(A) Silencing c-Kit expression is sufficient to inhibit c-Kit-EC-induced cell death. The HCT116 stable cell line inducible for c-Kit-EC was transfected with siCTL or c-Kit-specific siRNA (siKIT). 24 hr after transfection, cells were treated with Dox, and cell death was evaluated by AnnexinV and PI double staining using flow cytometry. Data are representative of three independent experiments.

(B and C) Deletion of the c-Kit gene protects cells from c-Kit-EC-induced apoptosis. The c-Kit gene was knocked down in the parental HCT116 cell line using the CRISPR/Cas9 technique and assessed by sgRNA-1 and sgRNA-2. The transgenic cell lines were then treated with conditioned medium obtained from stable HCT116 cells inducible for c-Kit-EC without (control) or with (c-Kit-EC) addition of Dox. Cell death was evaluated by AnnexinV and PI double staining (B) and DNA fragmentation (C) by flow cytometry.

(D–F) The HCT116 stable cell line inducible for c-Kit-EC was engrafted in SCID mice. Mice were intraperitoneally treated every day with Dox (50 mg/kg diluted in H<sub>2</sub>O) or vector. Tumor sizes were measured at the indicated days after xenograft (D) and after the mice were sacrificed (E). Photos of representative mice were taken right before sacrificing the mice (F). Tumors are indicated by red circles.

Data are representative of at least three independent experiments as mean ± SEM. \*p < 0.05.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-c-Kit	R&D	Cat#47233
Rabbit polyclonal anti-c-Kit	Enzo Life Science	Cat#ADI-KAP-TK005-E
Mouse monoclonal anti-c-Kit-PE	Miltenyi Biotec	Cat#130-099-672
Goat polyclonal anti-SCF	R&D	Cat#AF-255-NA
Mouse monoclonal anti- $\beta$ -actin (C4), HRP	Santa Cruz Biotechnology	Cat#sc-47778
Rabbit polyclonal anti-HA	Sigma-Aldrich	Cat#H6908
Rabbit polyclonal anti-caspase-3	Cell Signaling	Cat#9662
Mouse monoclonal anti-phospho-tyrosine	Santa Cruz Biotechnology	Cat#sc-508
Mouse monoclonal anti-ERK1/2	Sigma-Aldrich	Cat#SAB1305560
Rabbit polyclonal anti-phospho-ERK1/2	Sigma-Aldrich	Cat#SAB4301578
Mouse monoclonal anti-caspase-9	Santa Cruz Biotechnology	Cat#sc-73548
Rabbit polyclonal anti-Ku80	Cell Signaling	Cat#2753
<b>Bacterial and Virus Strains</b>		
Stellar Competent Cells, <i>E. coli</i> HST08 strain	Takara	Cat#636763
One Shot Stbl3 Chemically Competent <i>E. coli</i>	Life Technologies	Cat#C737303
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Lipofectamine LTX with Plus Reagent	Life Technologies	Cat#15338100
Lipofectamine RNAiMAX Transfection Reagent	Life Technologies	Cat#13778150
Jet PRIME	Polyplus-transfection	Cat#114-15
FuGENE HD Transfection Reagent	Promega	Cat# E2311
Protease inhibitor mixture	Roche Applied Science	Cat#04693116001
Phosphatase Inhibitor Cocktail 1	Sigma-Aldrich	Cat#p2850
Propidium iodide (PI)	Biotium	Cat#40017
Doxycycline hyclate	Sigma-Aldrich	Cat#D9891-5G
Ribonuclease A from bovine pancreas (RNaseA)	Sigma-Aldrich	Cat#R5503-3G
Imatinib mesylate	Sigma-Aldrich	Cat#SML1027-10MG
Protein G Sepharose 4 Fast Flow	GE Healthcare	Cat#10248260
Q-VD-OPh hydrate	Sigma-Aldrich	Cat# SML0063-1MG
Purified caspase-3	Guy Salvesen (The Burnham Institute, La Jolla, CA)	N/A
Caspase-3 Inhibitor Z-DEVE-FMK	R&D	Cat#FMK004
Human Stem Cell Factor (hSCF)	Cell Signaling	Cat#8925
Recombinant Human SCF Protein	R&D	Cat#255-SC-050
<b>Critical Commercial Assays</b>		
NucleoSpin RNAiI Kit	Macherey-Nagel	Cat#740955.240C
PrimeScript RT Reagent Kit	Takara	Cat#RR037B
APC Annexin V Apoptosis Detection Kit with PI	BioLegend	Cat#640932
Caspase-3 Fluorometric Assay Kit	BioVision	Cat#K105-400
Caspase-Glo 9 Assay Systems	Promega	Cat#G8211
DeadEnd Fluorometric TUNEL System	Promega	Cat#G3250
JC-1 Mitochondrial Membrane Potential Detection Kit	Biotium	Cat#30001
<b>Experimental Models: Cell Lines</b>		
Human: A549	ATCC	CCL-185
Human: HCT116	ATCC	CCL-247

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: IMR32	ATCC	CCL-127
Human: HMCB	ATCC	CRL-9607
Human: M2Ge	ATCC	RRID: CVCL_W291
Human: XPC	Centre Léon Bérard	N/A
Experimental Models: Organisms/Strains		
Female SCID mouse	Charles River	N/A
Recombinant DNA		
pITR plasmid vector	Prof. Rolf Marschalek, Goethe-University of Frankfurt, Germany	N/A
pSBtet-GH vector	Addgene	Cat#60498
Self-inactivating HIV-1-derived vector (pWPXLd)	Dr. Carine Maisse, INRA UMR754, Lyon, France	N/A
plentiCRISPR v2 vector	Dr. Carine Maisse, INRA UMR754, Lyon, France	N/A

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for reagents and primer sequences should be directed to the Lead Contact, Patrick Mehlen ([patrick.mehlen@lyon.unicancer.fr](mailto:patrick.mehlen@lyon.unicancer.fr)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell Lines**

Human lung A549 and colon HCT116 cancer cell lines were cultured in DMEM medium (Life Technologies) containing 10% fetal bovine serum. Human neuroblastoma IMR32 cell line was cultured in RPMI 1640 + Glutamax medium (Life Technologies) containing 10% fetal bovine serum. Human melanoma HMCB, M2Ge and XPC cell lines were cultured in DMEM + Glutamax supplemented with pyruvate (Life Technologies).

**Animal model**

Five-week-old (20-22 g body weight) female SCID mice were obtained from Charles River animal facility. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. Stable HMCB and HCT116 cell lines were implanted by subcutaneous injection of  $5 \times 10^6$  cells in 100  $\mu$ l of growth factors reduced matrigel (Corning) diluted in 100  $\mu$ l of PBS into the right flank of the mice. When tumors reached 100 mm<sup>3</sup> in approximately one week after injection, 1mg of doxycycline in 200  $\mu$ l or an equal volume of PPI water for control group was intraperitoneal injected daily for ten days. Tumor sizes were measured with a calliper. The tumor volume was calculated with the formula  $v = 0.5 \times (l \times w^2)$ , where v is volume, l is length, and w is width. All experiments were performed in accordance with relevant guidelines and regulations of animal ethics committee (Authorization no CLB-2015-004; accreditation of laboratory animal care by CECCAP, ENS Lyon-PBES, France).

**METHOD DETAILS**

**Transfection Procedures, Reagents**

For RNA interference experiments, cell lines were transfected using lipofectamine RNAiMAX reagent (Life Technologies). For plasmid transfection, A549 and IMR32 cell lines were transfected using lipofectamine Plus reagent (Life Technologies); HCT116 cell line was transfected using jet prime (PolyPlus-Transfection); HMCB cells were transfected with FuGENE HD (Promega). All transfections were performed according to the manufacturer's protocol. Recombinant SCF protein was obtained from R&D systems (E.coli-derived) or Cell Signaling (293T-derived).

**Generation of stable cell lines**

For the generation of inducible stable cell lines, flag-tagged human c-Kit coding sequence was cloned at Sfil sites in the pITR plasmid, a sleeping beauty-based vector allowing the doxycycline inducible expression of c-Kit or c-Kit-EC, as well as the constitutive expression of rtTA protein, puromycin resistance gene and green fluorescent protein (GFP), expressed under the control of the mouse phosphoglycerate kinase (PGK) promoter. Alternatively, c-Kit was cloned in pSB-tet plasmids, a second-generation of sleeping beauty-based vectors, containing an artificial promoter (RPBSA) to drive expression of rtTA protein, antibiotic resistance gene and fluorescent protein (Kowarz et al., 2015). A549, IMR32, HMCB and HCT116 cell lines were co-transfected

with c-Kit-pITR and a sleeping beauty transposase expression vector (SB100X). pITR vector was a generous gift of Prof. Rolf Marschalek (Goethe-University of Frankfurt, Germany). pSBtet-GH was a gift from Eric Kowarz (Addgene plasmid # 60498). Stable cell clones were selected for 5 days in puromycin or hygromycin-containing medium. Then wt and mutated c-Kit stable cell lines were sorted by flow cytometry to selected GFP positive cells, which are the stable inducible cells. All the stable inducible cell lines were further tested for c-Kit or c-Kit-EC expression in response to doxycycline.

Stable cell line constitutively expressing the intracellular region of c-Kit were generated by lentiviral infection, using self-inactivating HIV-1-derived vector (pWPXLd), encoding c-Kit (540-976 region) under the control of Human Elongation Factor-1 alpha (EF-1 Alpha) promoter. For the generation of stable cell line knocked-out for c-Kit or caspase-3 and -9, different small guide RNAs (sgRNA) were designed using the sgRNA designer tool (CRISPRko), available at <https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design> site. BsmBI linker were added to sgRNAs and the oligonucleotides were then annealed by incubation for 3min at 90°C and 15min at 37°C and ligated in plentiCRISPR v2 vector (Sanjana et al., 2014). Insertion of sgRNA was confirmed by sequencing. The use of plentiCRISPR v2, which is constructed around a 3rd generation lentiviral backbone, allows for the simultaneous infection/transfection of the vector for the expression of Cas9 and gRNA and for selection for puromycin resistance. Lentiviral vectors were a generous gift of Carine Maisse (INRA UMR754, Lyon, France).

### Quantitative RT-PCR

Total RNAs from cancer cell lines were extracted using NucleoSpin RNAII Kit (Macherey Nagel) according to manufacturer's protocol. Total RNAs from xenograft tumors were extracted by disrupting tissues with MagNALyser instrument (Roche Applied Science). RT-PCR reactions were performed with PrimeScript RT Reagent Kit (Takara). One micro gram total RNA was reverse transcribed using the following program: 37°C for 15min and 85°C for 5sec. For expression studies, the target transcripts were amplified in LightCycler2.0 apparatus (Roche Applied Science), using the Premix Ex Taq probe qPCR Kit (Takara), according to manufacturer instructions. Expression of target genes was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA binding protein (TBP) and phosphoglycerate kinase (PGK) genes, used as housekeeping genes. The amount of target transcripts, normalized to the housekeeping gene, was calculated using the comparative CT method. A validation experiment was performed, in order to demonstrate that efficiencies of target and housekeeping genes were approximately equal. The sequences of the primers are available upon request.

### Western Blotting Analysis

For immunoblotting analysis of c-Kit proteins, phospho-ERK and phosphor-c-Kit, and active caspase-3 stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% or 2% (for caspase activation) serum. Cells were then lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor (Sigma). For immunoblotting analysis of engrafted tumor cells, after sacrificing the mice, tumors were grinded and sonicated in RIPA lysis buffer in the presence of protease inhibitor mixture (Roche Applied Science).

Lysates were incubated 1h at 4°C and cellular debris were pelleted by centrifugation (10.000 g 15' at 4°C) and protein extracts (20-50µg per lane) were loaded onto 4%-15% SDS-polyacrylamide gels (Biorad) and blotted onto nitrocellulose sheets using Trans-Blot Turbo Transfer System (Biorad). Filters were blocked with 10% non-fat dried milk or 10% BSA in PBS/0.1% Tween 20 (PBS-T) for 1 hour and then incubated over-night with mouse monoclonal  $\alpha$ -c-Kit (dilution 1:2000, clone # 47233, R&D systems), rabbit polyclonal  $\alpha$ -c-Kit (1:2000, Enzo Life Science), goat polyclonal  $\alpha$ -SCF (1:1000, R&D systems), mouse monoclonal  $\alpha$ - $\beta$ -actin (1:1000, clone C4, HRP-conjugated, Santa Cruz Biotechnology), rabbit polyclonal  $\alpha$ -HA (1:2000, Sigma), rabbit polyclonal  $\alpha$ -caspase-3 (1:1000, Cell Signaling Technology), mouse monoclonal  $\alpha$ -phospho-tyrosine (1:1000, clone PY20, Santa Cruz Biotechnology), mouse monoclonal  $\alpha$ -phospho-ERK1/2 (1:1000, Sigma) and rabbit polyclonal  $\alpha$ -ERK1/2 (1:1000, Sigma) antibodies. After three washes with PBS-T, filters were incubated with the appropriate HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch, Suffolk, UK) for 1h. Detection was performed using West Dura Chemiluminescence System (Pierce). Membranes were imaged on the ChemiDoc Touch Imaging System (Biorad).

### Membrane staining of wild-type or mutated c-Kit

IMR32 c-Kit stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% serum. Cells were washed and detached using 0.53mM ethylenediaminetetraacetic acid (EDTA) in PBS.  $1 \times 10^5$  cells were collected for each condition and washed twice with 1ml PBFA (PBS-2% Foetal Bovine Serum-0.1% NaN<sub>3</sub>). Then cells were incubated with antibody anti-c-Kit conjugated with Phycoerythrin (PE) (CD117-PE, human, clone: AC126, Miltenyl Biotec) in 100µl PBFA buffer for 1 hour at 4°C. After being washed twice again by PBFA, cells were re-suspended in 300 µL cold PBS and were then assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur (BD Biosciences).

### In Vitro Transcription/Translation and Caspase Cleavage Reactions

Purified caspase-3 were a generous gift from Guy Salvesen (The Burnham Institute, La Jolla, CA). *In vitro* transcription/translation and incubation with caspases-3 were performed as described previously (Mehlen et al., 1998).

### Cell Death Assay

For propidium iodide (PI) staining, stable inducible cell lines were seeded in 96-well-plates at density of  $1.5 \times 10^3$  cells/well (A549) or  $3 \times 10^3$  cells/well (IMR32) or in 12-well-plates at density of  $1 \times 10^5$  cells/well (HMCB and HCT116). The following day, cells were treated with doxycycline and 0.33  $\mu\text{g/ml}$  PI in serum-free (A549) or serum-poor (2%) medium (IMR32, HMCB and HCT116) and the plates were transferred into Incucyte Zoom instrument (Essen Bioscience). Images of living cells and PI-stained dead cells were taken every two or three hours and PI-stained cell numbers were calculated.

For AnnexinV and PI double staining, cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMCB and HCT116) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 24 hours by doxycycline. Then cells were collected, washed with PBS and incubated for 15 min on ice in Annexin V buffer containing allophycocyanin (APC)-conjugated Annexin V and PI (BioLegend). Annexin V-positive cells were then assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur (BD Biosciences).

For DNA fragmentation analysis (SubG1), HMCB or HCT116 cell lines were plated onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well. The following day, cell medium was replaced by low-serum (2%) medium and cells were treated with doxycycline for 24 hours, 48 hours or 72 hours. For experiments combining SCF silencing and Imatinib treatment, M2Ge cells were plated onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well 24 hours before to be transfected with siRNAs. 24 hours after transfection, transfected cells were treated with Imatinib (Sigma) in non-serum medium for 24 hours. Then cells were collected and fixed in 70% ethanol and incubated at  $-20^\circ\text{C}$  for at least one night. Then, cells were washed twice with PBS and incubated for 15 min at room temperature in PBS containing 2 mg/ml RNase A and 40  $\mu\text{g/ml}$  PI. DNA content was evaluated by fluorescence activated cell sorting (FACS) analysis on a FACScalibur.

Caspase-3 activity assay was performed as described previously (Paradisi et al., 2009). Briefly, stable inducible cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMCB and HCT116) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 24 hours by doxycycline. Cells were collected and caspase-3 activity assay was performed using the Caspase-3 Fluorometric Assay Kit (BioVision), according to the manufacturer's instructions. Caspase activity (activity / min / mg of protein) was calculated from a 1h kinetic cycle reading on a spectrofluorometer (405nm / 510nm, Infinite F500, Tecan).

TUNEL-PI double staining assay was performed using DeadEnd Fluorometric TUNEL System (Promega), according to manufacturer protocol. Briefly, HCT116 cells were seeded onto 6-well-plates at a density of  $1.5 \times 10^5$  cells/well. 24 hours after, cell medium was replaced by low-serum (2%) medium and cells were treated with doxycycline for further 48 hours or 72 hours. Then, cells were collected and fixed in 5ml of 1% methanol-free formaldehyde for 20 minutes on ice. After washing twice with PBS, cells were permeabilized by ice cold 70% ethanol for 24 hours and then washed again twice with PBS. Finally, cells were stained with fluorescein-12-dUTP and PI and TUNEL staining and DNA content was assessed by fluorescence activated cell sorting (FACS) analysis on a FACScalibur.

### Immunoprecipitation (IP) and caspase-9 assay

Stable inducible cell lines were treated with doxycycline for 24 hours in medium containing 10% serum. Alternatively, 293T cells were transfected with both kinase-mutated or double-mutated c-Kit and caspase-9 plasmids, using Fugene HD (Roche Applied Science) and according to manufacturer instructions. Cell culture dish was placed on ice and cells were washed with ice-cold PBS (Phosphate buffered saline), and ice-cold lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 0.1% Nonidet P-40 (NP-40), 2 mM EDTA) was added on to the cells. Adherent cells were scraped off the dish then cell suspension was transferred gently into a microcentrifuge tube, sonicated and maintained at  $4^\circ\text{C}$  for 1 hour. Lysates were then centrifuged at 17,000 x g for 30 minutes at  $4^\circ\text{C}$  and the supernatants were transferred in a fresh tube kept on ice. Protein concentration was measured and the same amount of protein was used for each sample. Lysates were incubated with 10  $\mu\text{l}$  of protein G-agarose beads for 30 min at  $4^\circ\text{C}$  with gentle rotation and then spinned in microcentrifuge at 5,000 x g for 5 min at  $4^\circ\text{C}$ , and precleared supernatants were incubated with  $\alpha$ -flag antibody (1:100 dilution) overnight at  $4^\circ\text{C}$  under gentle rotation. Next, 50  $\mu\text{l}$  protein G-agarose beads was mixed with each sample and incubated for 2 hours at  $4^\circ\text{C}$  with gentle rotation. After the incubation, the mixture was then centrifuged, supernatant removed from the beads and discarded. Beads were washed with lysis buffer five times to remove non-specific binding. For each wash, beads were mixed gently with wash buffer, centrifuged at  $4^\circ\text{C}$  and the supernatant was then discard. After final wash, beads were resuspended in 50  $\mu\text{l}$  lysis buffer, and the elution/beads mixture was used for caspase-9 assay, using the Caspase-Glo 9 Assay Kit (Promega), according to the manufacturer's instructions. Luminescence, proportional to caspase-9 activity, was recorded on the Infinite F500 luminometer (Tecan). For western blot, lysates from 293T cells transfected with Caspase-9-HA and inducible kinase-mutated c-Kit were immunoprecipitated with  $\alpha$ -HA antibody (1:100, Sigma), incubated overnight at  $4^\circ\text{C}$ . After addition of protein G-agarose beads and incubation for 2 hours at  $4^\circ\text{C}$ , pulled-down proteins were eluted in laemmli sample buffer 2x. After incubation at  $95^\circ\text{C}$  for 10 minutes and a brief spin, immunoprecipitated proteins were resolved by SDS-PAGE and pulled-down c-Kit was detected using mouse monoclonal  $\alpha$ -c-Kit antibody (1:2000, R&D systems).

### Mitochondrial potential assay

Stable inducible cells were seeded onto 6-well-plates at a density of  $2 \times 10^5$  cells/well (HMCB) or  $3 \times 10^5$  cells/well (IMR32). The following day, cell medium was replaced by low-serum (2%) medium and cells were treated for 36 hours with doxycycline. Then cells were collected, washed with PBS.  $2 \times 10^5$  (HMCB) or  $4 \times 10^5$  (IMR32) cells for each sample were suspended in 200  $\mu\text{l}$  buffer containing

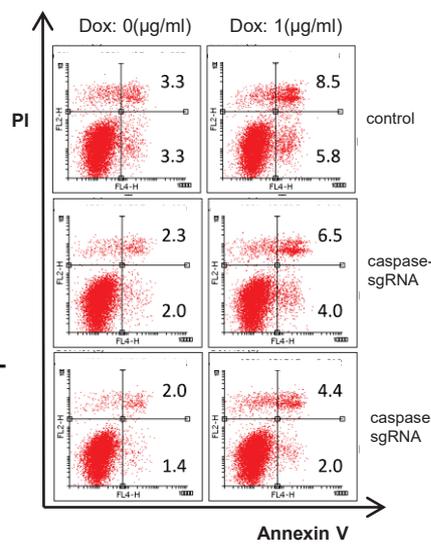
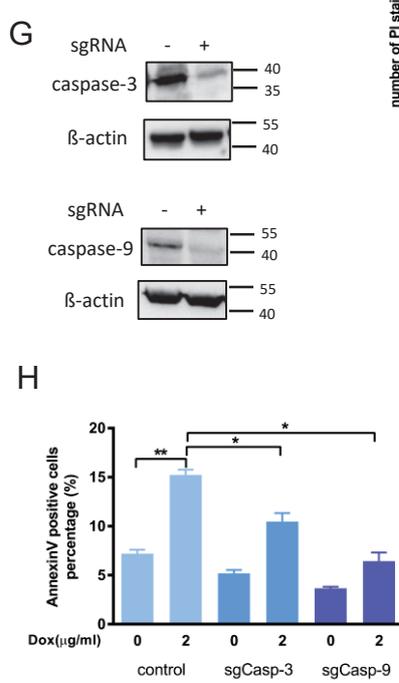
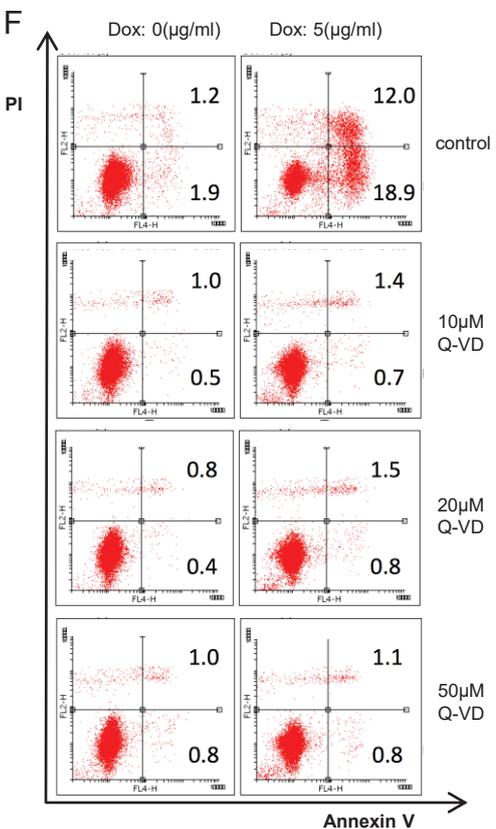
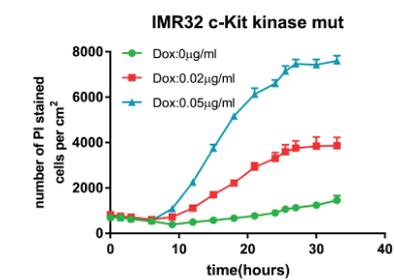
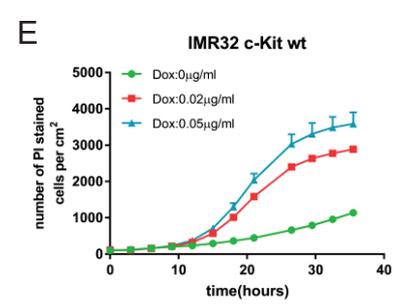
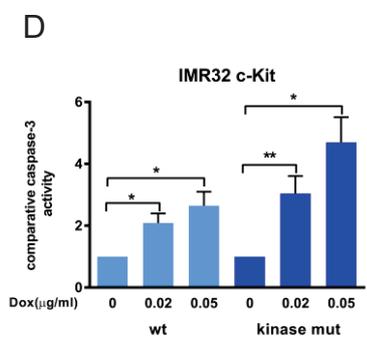
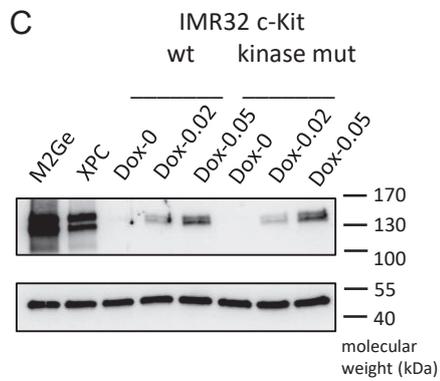
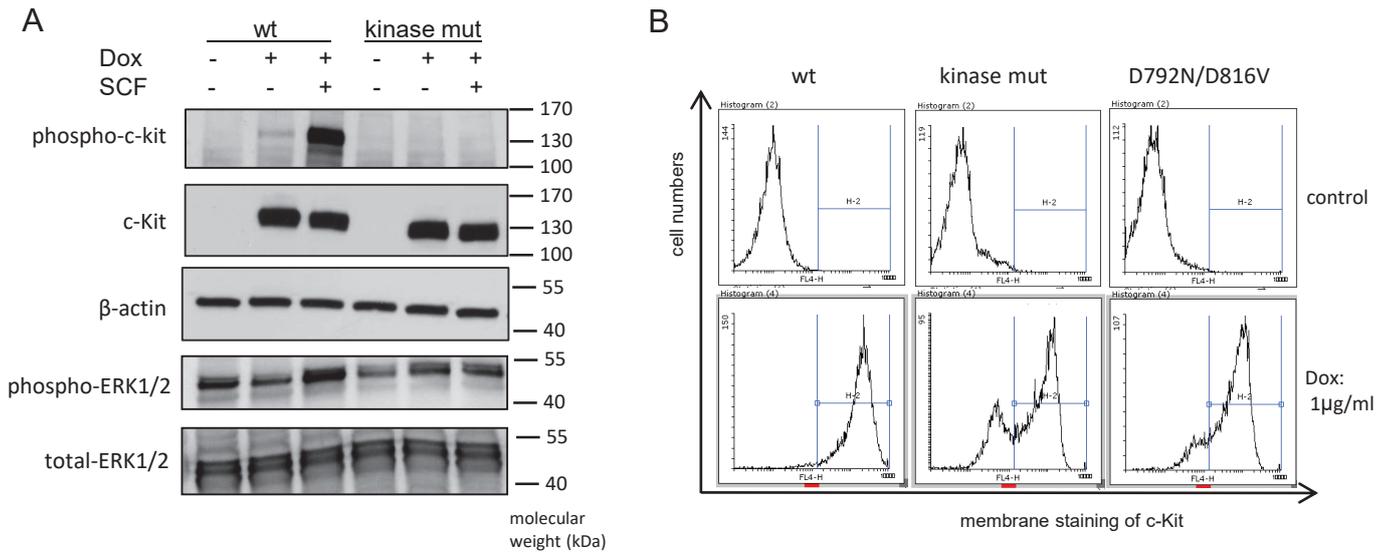
JC-1 dye (Biotium). After incubation at 37°C for 15 minutes, stained cells were centrifuged at 800 x g for 5 min at room temperature and supernatant was removed completely without disturbing the cell pellet. Afterward, cell pellet was resuspended in 1 mL PBS for washing, then centrifuged at 800 x g for 5 min at room temperature and supernatant was removed again. Washing step was repeated once. Later, cell pellet was resuspended by pipetting in 50 $\mu$ l PBS buffer containing 1  $\mu$ g/ml DAPI. The final stained cells were analyzed by using NucleoCounter NC-3000 (Chemometec).

#### **Conditional medium production**

HCT116 stable cells inducible for c-Kit-EC were plated in 75 cm<sup>2</sup> dishes (2.5\*10<sup>6</sup> cells/dish). The following day, cell medium was replaced with 8ml serum-free medium and cells were treated for 24 hours with or without doxycycline. Collected cell media were centrifuged at 1200Xg for 5 minutes and supernatant was recovered as c-Kit-EC or control conditional medium.

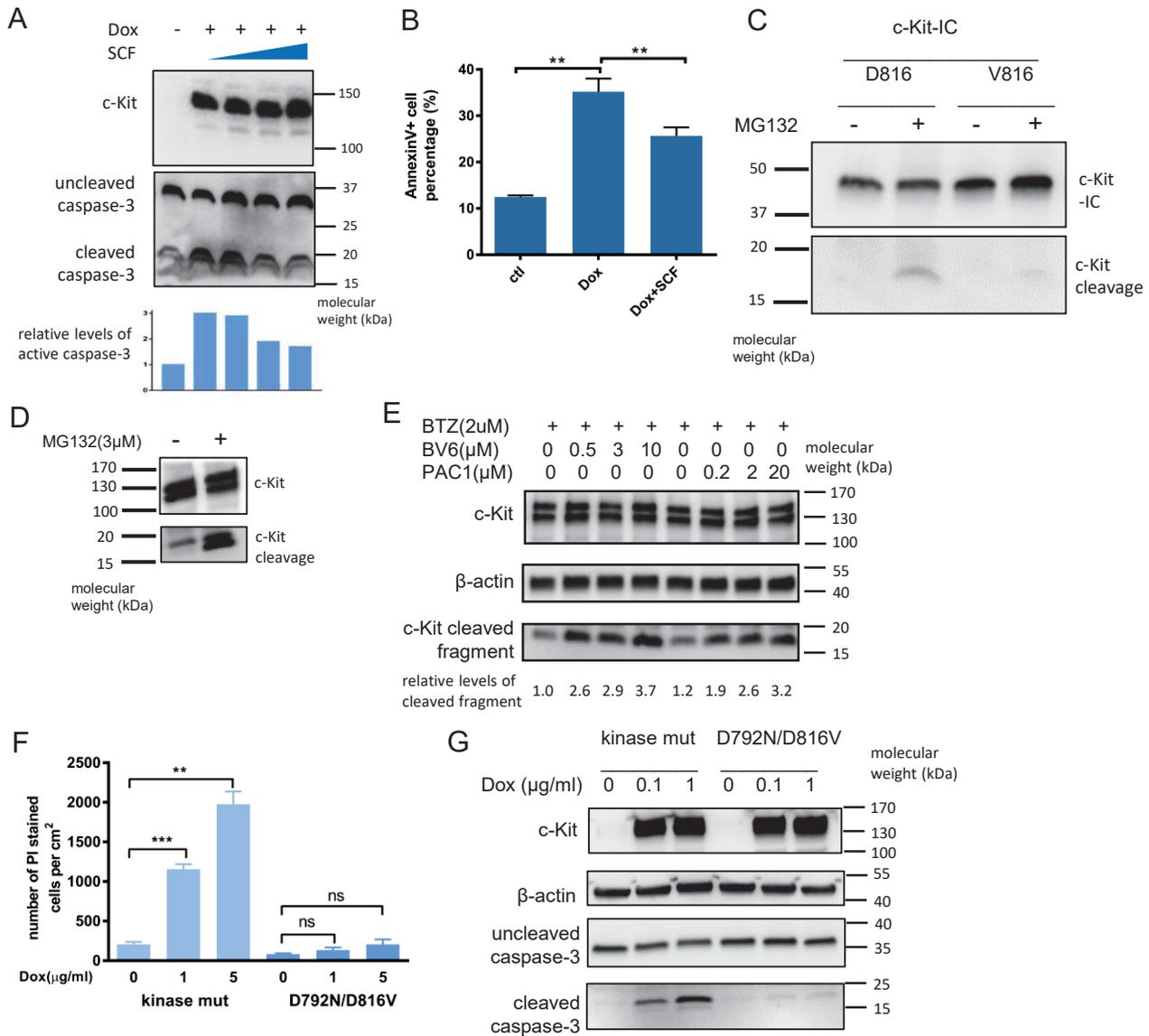
#### **QUANTIFICATION AND STATISTICAL ANALYSIS**

The data reported are the mean  $\pm$  SEM of at least three independent determinations, each performed in triplicate. Statistical analysis was performed by the Student's t test.



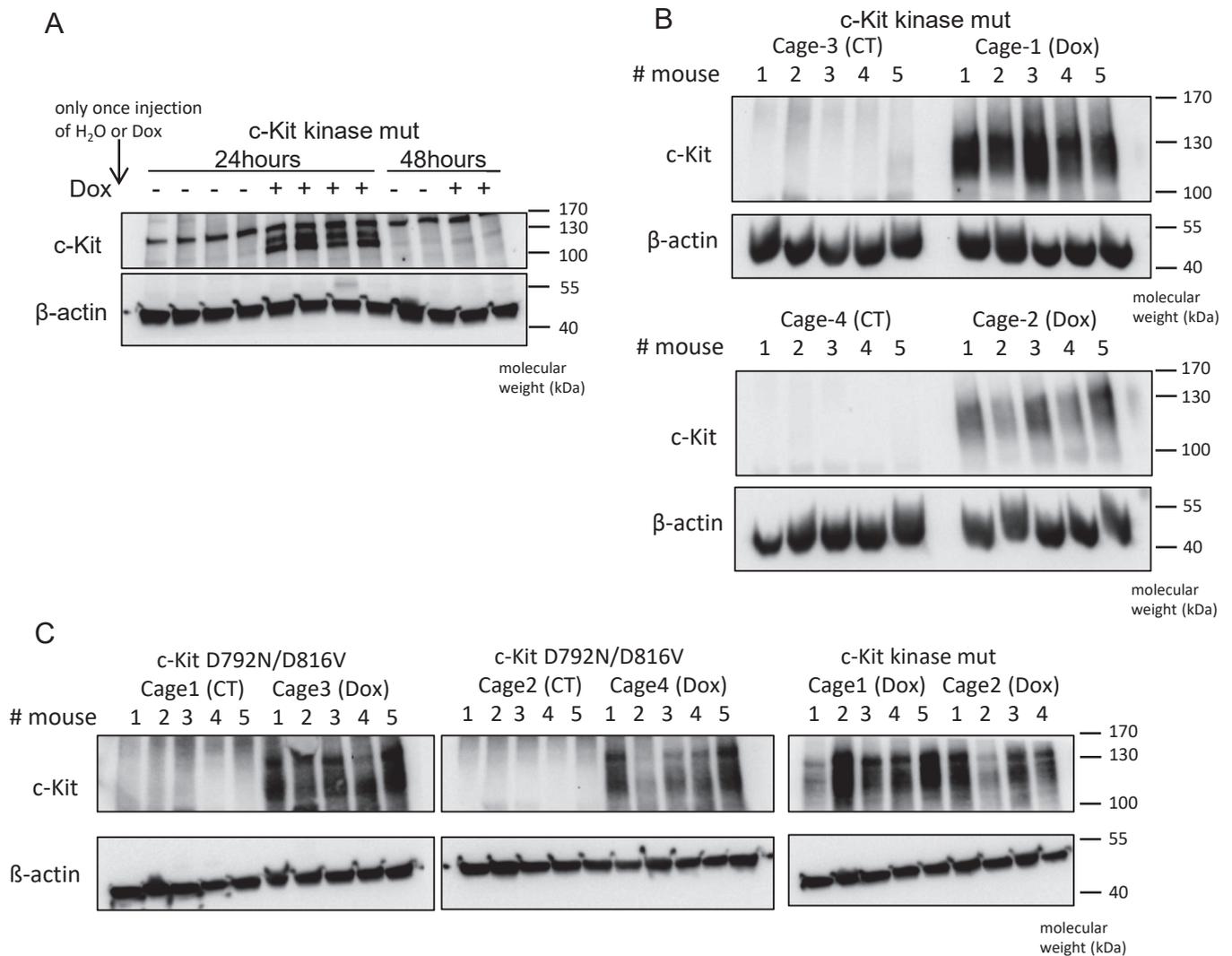
**Figure S1: c-Kit induces caspase-dependent apoptosis *in vitro*. Related to Figures 1,2.**

**(A):** Kinase-mutated c-Kit cannot be activated by SCF. A549 stable cells inducible for wild-type (wt) or kinase-mutated (kinase mut) c-Kit were treated with doxycycline in the presence or in the absence of SCF. c-Kit activity was evaluated by its ability to be autophosphorylated in presence of SCF, as well as by phosphorylation of ERK1/2. **(B):** Over-expressed wild-type and mutant c-Kit proteins are expressed in cellular membrane. IMR32 stable cell line inducible for wild-type (wt) kinase-mutated (kinase mut) and kinase- and caspase-mutated (D792N/D816V) c-Kit was treated with doxycycline for 24 hours. Membrane staining of c-Kit proteins was then performed by flow cytometry of non-permeabilized cells. **(C):** c-Kit stable inducible cell lines express endogenous-like protein levels. Wild-type (wt) and kinase-mutated (kinase mut) c-Kit were induced in IMR32 stable inducible cells by adding 0.02 or 0.05 $\mu$ g/ml of doxycycline, and protein levels were compared to endogenous c-Kit expressed in M2Ge and XPC melanoma cells. Western blot was performed using a c-Kit antibody targeting the C-terminal region of the receptor. **(D,E):** Ectopic c-Kit expression induced by low doxycycline concentration is sufficient to trigger apoptosis. Stable IMR32 cells were treated with the indicated Doxycycline concentrations for 6 hours and caspase activity was evaluated by fluorometric detection kit (D). Cell death induction by low-expressed ectopic c-Kit was confirmed by PI staining measured by Incucyte Zoom device (E). **(F):** c-Kit-induced cell death requires active caspase-3. HMCB stable cell line inducible for kinase-mutated c-Kit were treated with doxycycline in presence of increasing concentrations of the pan-caspases inhibitor Q-VD-OPh (Q-VD). Apoptotic rate was evaluated by AnnexinV/PI double staining by flow cytometry. **(G,H):** Deletion of caspase-3 and caspase-9 protects cells from c-Kit-induced apoptosis. Caspase-3 and caspase-9 were knocked-down in IMR32 stable cells inducible for kinase-mutated c-Kit, using CRISPR-Cas9 technique. The knocking-down efficiency was evaluated by western blot (G). Cell death induction upon doxycycline treatment in control and caspase knocked-down cell lines was evaluated by AnnexinV/PI double staining using flow cytometry (H). In the left panel, a representative experiment is presented, while in the right panel the combined result of three independent experiments is shown. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; Dox, doxycycline; sgRNA, small guide RNA.



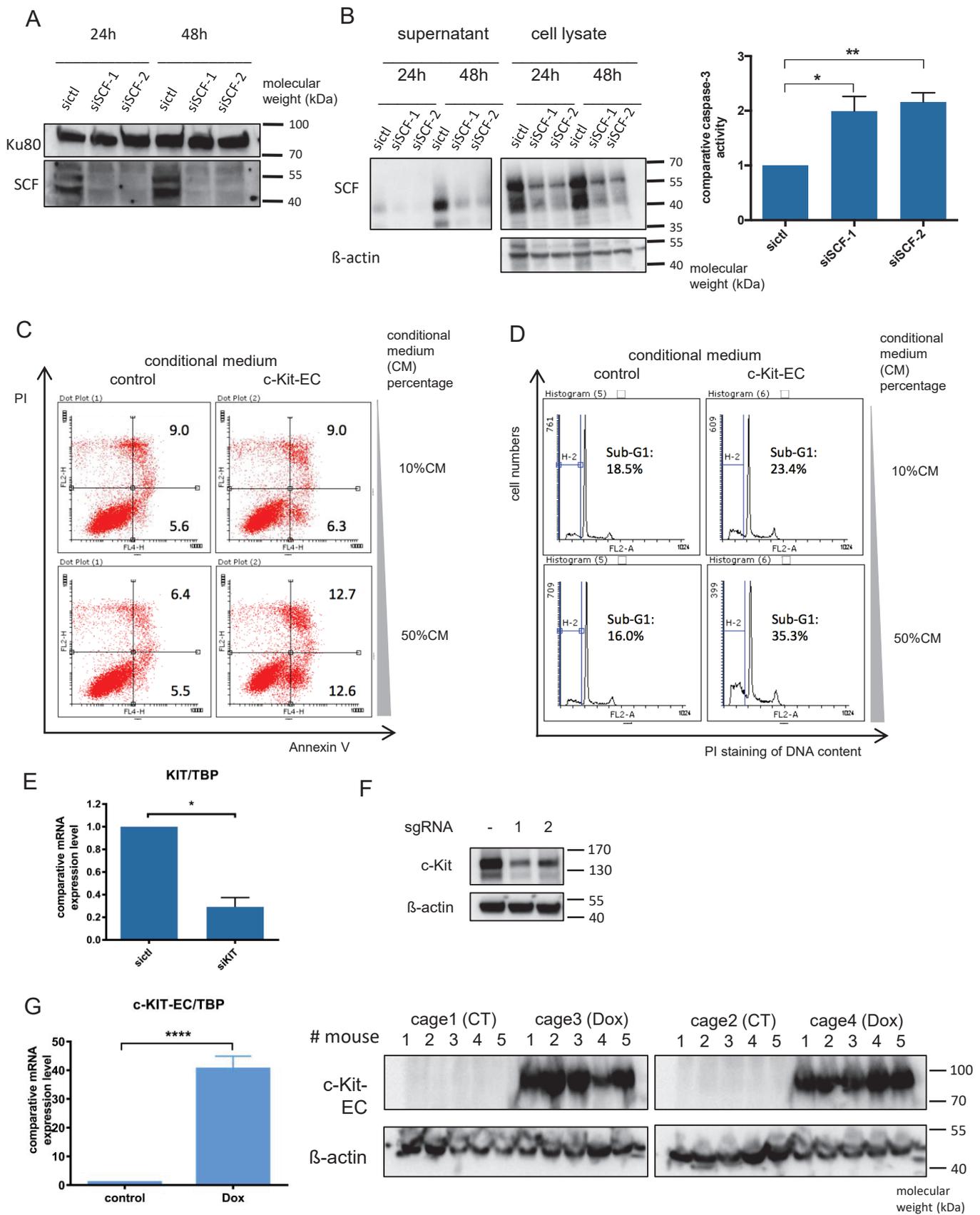
**Figure S2: c-Kit-induced apoptosis requires SCF absence and c-Kit caspase cleavage. Related to Figures 3,4.**

**(A,B):** The ligand SCF inhibits c-Kit-induced cell death. IMR32 stable cell lines inducible for kinase-mutated c-Kit were treated with doxycycline and increasing concentrations of recombinant SCF protein (supplied by R&D systems). Apoptotic cell death was then evaluated by caspase-3 activation, assessed by caspase-3 cleavage revealed by western blot (A) and by AnnexinV/PI double staining (B), analyzed by flow cytometry (C): c-Kit is cleaved at D816 residue site. Stable A549 cells expressing HA-tagged wild-type (D816) or caspase-mutated (V816) intracellular (IC) c-Kit fragment were treated with the proteasome inhibitor MG132 and the generation of c-Kit cleavage band was evaluated by western blot. **(D):** cleavage of endogenous c-Kit. M2Ge melanoma cells were treated for two hours with MG132, and the generation of 18kDa-fragment was evaluated by western blot, using a c-Kit antibody targeting C-terminal region. **(E):** caspase activation promotes c-Kit cleavage. M2Ge cells were treated for six hours with increasing concentrations of caspase activators BV-6 and PAC1. Four hours after, cells were treated with the proteasome inhibitor Bortezomib (BTZ) for further two hours. c-Kit cleaved fragment was then revealed by western blot. **(F,G):** c-Kit caspase cleavage is required for cell death induction. A549 and IMR32 stable cell lines inducible for kinase-mutated (mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit were treated with the indicated doxycycline concentrations for 48 hours. Apoptotic cell death was then evaluated by both PI staining in A549 cells, using Incucyte Zoom instrument (E) and caspase-3 cleavage by western blot in IMR32 cells (F). *ns*, non-significant; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Dox, doxycycline; BTZ, Bortezomib; PI, propidium iodide; IC, intracellular region.



**Figure S3: *In vivo* model with c-Kit expression in engrafted tumors. Related to Figure 5.**

**(A,B,C):** Exogenous c-Kit is induced in engrafted cells upon doxycycline treatment. SCID mice were engrafted with HMCB stable cells inducible for kinase-mutated (kinase mut) or kinase- and caspase-mutated (D792N/D816V) c-Kit. To study c-Kit stability after induction with doxycycline, mice were sacrificed 24 hours or 48 hours after intraperitoneal injection with 50mg/kg of doxycycline and c-Kit expression was evaluated by western blot on engrafted tumors (A). Then, kinase-mut (B,C) or D792N/D816V (C) c-Kit protein was assessed by western blot on engrafted tumors obtained from SCID mice treated every day with 50 mg/kg of doxycycline for 10 days. Engrafted tumors were grinded and sonicated in RIPA buffer. Dox, doxycycline; CT, control.



**Figure S4: SCF silencing or interference triggers c-Kit-dependent apoptosis and limits tumor growth. Related to Figures 6,7.**

**(A):** SCF protein expression in siRNA-transfected A549 cell line. Cells were transfected with scramble (sictl) or two SCF-specific (siSCF-1 and siSCF-2) siRNAs. The efficiency of gene silencing was evaluated by western blot. Lupus Ku autoantigen protein p80 (Ku80) was used as housekeeping protein. **(B):** Silencing SCF induces caspase-3 activation in HCT116 cell line. Cells were transfected with scramble (sictl) or SCF-specific (siSCF-1 and siSCF-2) siRNAs. The efficiency of SCF silencing was assessed by western blot (left) 24 hours and 48 hours after transfection. Caspase-3 activation (right) was measured 24 hours after transfection using the fluorometric detection kit. **(C,D):** Conditional medium containing c-Kit-EC induces apoptosis in parental HCT116 cell line. HCT116 stable cell line inducible for c-Kit-EC was treated with doxycycline (c-Kit-EC) or water (control). 24 hours later, conditioned medium (CM) was diluted in normal medium at the indicated concentrations and used to treat parental HCT116 cells. Apoptosis was then evaluated by AnnexinV/PI double staining (C) or DNA fragmentation (D) by flow cytometry. **(E):** KIT gene expression in siRNA-transfected HCT116 stable cell line. Cells were transfected with scramble (sictl) or KIT-specific siRNA (siKIT). The efficiency of gene silencing was evaluated by quantitative PCR, normalized with TBP gene expression. **(F):** c-Kit was knocked-down in XPC melanoma cell line by CRISPR-Cas9 technique, using two c-Kit specific guide RNAs. The knocking-down efficiency was evaluated by western blot. **(G):** SCID mice were engrafted with HCT116 stable cells inducible for c-Kit-EC and treated daily with 50 mg/kg of doxycycline. After 10 days, mice were sacrificed and total RNA and protein were extracted from engrafted tumours. c-Kit-EC mRNA following doxycycline treatment, normalized with TBP gene expression, was then measured by quantitative PCR (left), while c-Kit-EC protein levels were assessed by western blot (right). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.0001$ . Dox, doxycycline; EC, extracellular domain; CM, conditional medium; TBP, TATA Binding Protein.