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Yan Sun

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Yan Sun. Regulation of Netrin-1 by p53 isoforms. Cellular Biology. Université de Lyon, 2020. English.  
NNT : 2020LYSE1058 . tel-03310180

**HAL Id: tel-03310180**

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N°d'ordre NNT : 2020LYSE1058

**THESE de DOCTORAT DE L'UNIVERSITE DE LYON**  
opérée au sein de  
**l'Université Claude Bernard Lyon 1**

**Ecole Doctorale ED 340**  
**(Biologie Moléculaire Intégrative et Cellulaire)**

**Spécialité de doctorat : Biologie Moléculaire et Cellulaire**

Soutenue publiquement le 24/03/2020, par :

**Yan SUN**

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**Régulation de la Nétrine-1 par les isoformes de p53**

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Devant le jury composé de :

BERNET Agnès	Professeure des Universités	Université de Lyon UMR5286 - CRCL	Présidente
GAIDDON Christian	Directeur de Recherche	Université de Strasbourg - U1113	Rapporteur
THIBERT Chantal	Chargée de Recherche	Institute for Advanced Biosciences – Grenoble U1209 - UMR5309	Rapporteur
MARCEL Virginie	Chargée de Recherche	Université de Lyon UMR5286 - CRCL	Examinatrice
TULASNE David	Directeur de Recherche	Institut de biologie de Lille – UMR8161	Examineur
FOMBONNE Joanna	Maître de Conférences	Université de Lyon UMR5286 - CRCL	Directrice de thèse
MEHLEN Patrick	Directeur de Recherche	Université de Lyon UMR5286 - CRCL	Invité
PARADISI Andrea	Chargé de Recherche	Université de Lyon UMR5286 - CRCL	Co-directeur de thèse

# Abstract

Netrin-1, a secreted protein recently characterized as an innovative cancer therapeutic target, is the anti-apoptotic ligand of Deleted in Colorectal Cancer (DCC) and UNC5H family member receptors, belonging to the dependence receptor family. As Netrin-1 is strongly expressed in several aggressive cancers, by inhibiting cell death induced by its receptors, interference of the binding with its receptors has been shown to actively induce apoptosis and tumour regression. To this line, a monoclonal neutralizing anti-Netrin-1 antibody has been developed and it is currently in phase I clinical trial. Recently, it has been shown that the transcription factor p53 positively regulates Netrin-1 gene expression.

Here we show that Netrin-1 up-regulation requires transcriptional activity of the second p53 transactivation domain. Moreover, we propose that Netrin-1 could be a new target gene of N-terminal p53 isoform  $\Delta 40p53$ , independently by full-length p53 activity. We demonstrate this hypothesis using stable cell lines, harbouring wild-type or null-p53, in which  $\Delta 40p53$  expression could be finely tuned. Our results indicate that  $\Delta 40p53$  is able to bind and activate Netrin-1 promoter, suggesting the formation of a  $\Delta 40p53$  homo-tetramer complex. In addition, employing CRISPR/Cas9 method, we show here that forcing immortalized human skeletal myoblasts cells to produce  $\Delta 40p53$  isoform, instead of full-length p53, leads to Netrin-1 and its receptor UNC5B up-regulation. As a consequence, cells increase Netrin-1 dependence to survival, allowing Netrin-1 inhibition to trigger apoptotic cell death. Finally, we clearly indicate a positive correlation between Netrin-1 and  $\Delta 40p53$  gene expression in human melanoma and colorectal cancer biopsies.

In conclusion, we propose here a pro-tumour role of  $\Delta 40p53$  through direct activation of Netrin-1, independently to full-length p53 activity. Following dependence receptor notion, inhibition of Netrin-1 binding to its receptors, instead of targeting dominant-negative function, should be a promising therapeutic strategy in human tumours expressing high levels of  $\Delta 40p53$ .

# RESUME

La nétrine-1 est une protéine sécrétée initialement découverte comme un indice de navigation des axones pendant le développement du système nerveux. Cependant, la Netrin-1 a récemment été impliquée dans la régulation de la tumorigenèse ; en effet, les principaux récepteurs de la Netrin-1, les membres de la famille DCC et UNC5, appartiennent à la famille émergente des récepteurs de dépendance. Ces récepteurs ont la particularité d'être également actifs en l'absence de leurs ligands, induisant une "signalisation négative" qui déclenche l'apoptose. Les récepteurs de dépendance sont des candidats suppresseurs de tumeurs. L'hypothèse générale est que ces récepteurs limitent le développement des tumeurs par l'induction de l'apoptose des cellules tumorales qui se développeraient ou migreraient au-delà des régions de disponibilité des ligands. Par conséquent, la transformation tumorale est associée à l'inhibition constitutive des signaux de mort induits par ces récepteurs, qui pourrait être réalisée, par exemple, par une régulation positive des ligands. En effet, un gain de Netrin-1, corrélé comme un avantage sélectif pour la progression tumorale, a récemment été décrit dans plusieurs cancers humains. Cependant, on sait peu de choses sur la régulation de l'expression de la Netrine-1 pendant le développement de la tumeur.

Le facteur de transcription p53 est composé de plusieurs domaines, tels que deux domaines de transactivation différents TAD1 et 2, un domaine de liaison à l'ADN et un domaine d'oligomérisation. Il a été démontré que les domaines de transactivation p53 régulent de manière différentielle des ensembles de gènes distincts. Pour mieux caractériser la régulation de la Nétrine 1 par p53, nous avons d'abord vérifié si la régulation de la Nétrine 1 par p53 nécessite une activité transcriptionnelle, et finalement pour identifier quel domaine de transactivation est nécessaire pour réguler la Netrine-1 à la hausse. Nous avons généré plusieurs lignées stables de mutation transactivationnelle p53 inductibles pour les différents mutants TA, et l'analyse de l'expression de la Netrine-1 par Western blot a révélé une forte augmentation de la Netrine-1 en présence de p53 de type sauvage, et aucun changement lors de l'inactivation de TA1. Cependant, l'inhibition de l'activité transcriptionnelle de TA2 empêche complètement la régulation à la hausse



de la Netrine-1 lors de l'induction de p53, ce qui indique que la régulation de la Netrine-1 nécessite l'activité transcriptionnelle de p53 TA2.

Récemment, nous avons montré que la Nétrine-1 et son récepteur principal UNC5B pouvaient être régulés par le facteur de transcription p53. Le gène TP53 peut être exprimé sous 12 isoformes différentes (p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 40p53 $\alpha$ ,  $\Delta$ 40p53 $\beta$ ,  $\Delta$ 40p53 $\gamma$ ,  $\Delta$ 133p53 $\alpha$ ,  $\Delta$ 133p53 $\beta$ ,  $\Delta$ 133p53 $\gamma$ ,  $\Delta$ 160p53 $\alpha$ ,  $\Delta$ 160p53 $\beta$ , and  $\Delta$ 160p53 $\gamma$ ) par l'initiation alternative de la traduction, l'utilisation de promoteurs alternatifs et/ou l'épissage alternatif. Les isoformes p53 sont exprimées différemment selon les types de cancer et elles ont également des activités transcriptionnelles et des fonctions suppressives de tumeur différentes qui peuvent affecter diverses autres fonctions biologiques.

Notre deuxième question est donc que ces isoformes deltaNp53 fonctionnent de manière similaire aux mutants TA, capables de réguler l'expression de la Netrin-1 et de son récepteur UNC5B, et de tester si elles pourraient s'influencer mutuellement pour équilibrer finement l'expression des gènes.

Nous produisons des lignées cellulaires stables exprimant principalement les isoformes p53, et il est intéressant de noter que 40p53 est capable de réguler l'expression de la Nétrine-1 et de UNC5B au niveau de la transcription et des protéines, d'une manière comparable à p53. En surexprimant les principales isoformes de p53, nous avons également constaté que  $\Delta$ 40p53 $\alpha$ , indépendamment du statut de p53, régule l'expression de la Netrin-1 et de l'UNC5B, comme nous avons pu l'observer dans les lignées de cellules cancéreuses de type sauvage ou nul pour p53.

De plus, grâce à un test de luciférase et à un test d'immunoprécipitation de la chromatine, nous avons montré que 40p53 se lie directement au promoteur de la Netrin-1 et l'active.

Enfin, pour confirmer la régulation positive de la Netrine-1 également par le  $\Delta$ 40p53 $\alpha$ , produit de manière endogène, nous avons utilisé les cellules myoblastes humaines immortalisées LHCN. Nous avons utilisé deux ARN guides différents pour cibler le premier ATG de la longueur complète de p53, le numéro guide 10, et l'ATG en position 40 correspondant au premier acide aminé de  $\Delta$ 40p53, en utilisant le numéro guide 400. Nous avons ensuite généré des lignées cellulaires LHCN stables en utilisant à la fois le guide et nous avons analysé l'expression de

p53 et de Netrin-1. Étonnamment, dans les cellules LHCN générées avec le numéro de guide 10, nous avons perdu la bande correspondant à la longueur complète de p53, mais une bande correspondant au poids moléculaire de  $\Delta 40p53$  est apparue. Cette bande a disparu lorsque nous avons utilisé le numéro de guide 400, et ensemble à l'observation que cette bande n'est pas reconnue par l'anticorps DO-1, ne reconnaissant que la longueur complète de p53, cela suggère qu'elle correspond à la protéine  $\Delta 40p53$ . Ensuite, l'utilisation de ces ARN guides nous permet de générer des lignées cellulaires stables exprimant le  $\Delta 40p53$  endogène. L'analyse de l'expression génétique montre une forte expression de la Netrin-1 et de l'UNC5B dans les cellules générées avec le numéro de guide 10, exprimant la delta40 endogène, par rapport aux cellules témoins mais aussi aux cellules générées avec le numéro de guide 400, qui sont complètement KO pour toutes les isoformes p53.

Enfin, nous avons observé une corrélation positive entre  $\Delta 40p53$  et l'expression de Netrin-1 dans les biopsies de mélanomes et de tumeurs colorectales, confirmant que  $\Delta 40p53$  pourrait être un biomarqueur des tumeurs à forte expression de Netrin-1.

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## **Acknowledgement**

First of all, I would like to thank all the jury member BERNET Agnès, GAIDDON Christian, THIBERT Chantal, MARCEL Virginie, TULASNE David, FOMBONNE Joanna. Thank you all of you for accepting my invitation, and thank you for your precious time reading my manuscript. I know some of you coming far from Lyon, thank you very much again for coming to my defense. It's my great honor to report my PhD project and discuss the results with you.

I would like to thank Patrick for all these three years guidance and help. Thank you for accepting me into your lab, which is really wonderful. I have impression that I was really lack of experience when I come 3 years ago, thank you for your trust and patience, thank you very much for giving me opportunities to join so many international conferences. I have learned a lot here, and all of these skills, technology and knowledge I learned here will definitely help me in the future career.

Thank you, Andrea, my best professor. It's really lucky to be your student. During these three years, whenever I have problems, I would always first think of you, and you were always there offering help and advices to me. I still remember, when I first work with you, I cannot even design my own experiments by myself, always lack of control group. Thank you for your explanation and guidance. Thank you for bring me to so many great international conferences, especially the 4th International p53 Isoforms Meeting in Dubrovnik. All these three years, you helped me fill in a lot of administrative excel files, documents, without you, I will not be able to finish my PhD. Thank you so much!

I would also thank Carine and Lucie, thank you very much for offering me the LHCN cells and prepared all the medium. It's a wonderful meeting with you in Croatia. And best wishes to Lucie for her defense.

I would also thank David, Melissa and Cathy. David and Melissa, you are always making company with me in the late night at lab. Because of you working around me, I was less lonely. Thank you, Cathy, every time when I lost myself in looking for something in the lab, you are always the first one coming to help me. It's really kind of you!

I would also thank my best partners, Ambroise and Hong, we work together for several years, I learned a lot from them: keep strong and never give up. We spent most of the time working in the same group, discussing the results, life, politics and culture difference. Many thanks to them, they really helped me a lot not only on experiments but also on all kinds of small problems in daily life. And thank you for giving me motivation on every difficult moment.

I would also thank to all the PhDs, masters and technician friends in our lab. Thank them for sharing with me all the interesting opinions on different topic about culture, languages, habits and life. They help me better understand the culture and life in France. I met them at different period, some of them have already graduated and left our lab, but all of them are so kind and nice that I will keep them in my heart forever. Many thanks to them: Anne-Rita, Mathieu, Verena, Lisa, Thomas, Lucas, Sarah, Shan, Stephany, Justine, Anna, Shuheng, Jeromine, Giacomo, Duygu, Laura, Clara, Guilhem, Thibaut...I wish you all good luck and happiness in your life.



I would also thank my dear colleagues Joanna, Benjamin, Nico, Olivier, Agnès, Gabriel, Amina, Peggy, Céline, Ficus, David N, Jennifer, Mitsuaki...They are all very professional researchers and post-docs. Thank you very much for all these years' help to my research and daily life. It's great pleasure to work with all them. Wish you have great progress in your research and also happiness in life in future.

I would like to thank all the administrative secretaries Yohann, Bérangère, Sophie, Quynh, Claire. Thank you for your kind help and reminds, so that all my registration processes are becoming easier and faster. Especially many thanks to Yohann, I have bothered you so many times, but you are always very patient with me, answering questions and helping me struggled in the complicated administrative system, thank you a lot.

Last but not least, I have to thank my family and all my friends: Anne-Marie, Michele, Swarna, Mahfoud, Yujie, Dianru, Mingyan, Hanli, Yaqi, Donglei, Xingjie, Minmin, Jummei, Jiaojiao...thank you for all my friends and family member to support and encourage me so much. I love you all!

## **Abbreviations**

ADDs	addiction/dependence domains
AML	acute myeloid leukemia
Apaf-1	apoptotic peptidase activating factor-1
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related protein
AURKA	aurora kinase A
Bak	Bcl-2 antagonist/killer-1
Bax	Bcl-2-associated X protein
BH	Bcl-2 homology
BIR	baculovirus IAP repeat
CARD	caspase recruitment domain
Caspases	cysteine-aspartic proteases
CCA	cholangiocarcinoma
CDK	cyclin-dependent kinase
CDON	cell-adhesion molecule-downregulated by oncogenes
CHK	checkpoint kinase
Chk1	Checkpoint Kinase1
CK	casein kinase
CSN-K	COP9 signalosome associated kinase complex
DAPk	Death-Associated-Protein kinase
DBD	DNA-binding domain
DCC	deleted in colorectal carcinoma
DD	death domains
DED	death-effector domain
DISC	death-inducing signaling complex
DNAPK	DNA-dependent protein kinase
DR	dependence receptor

DRAL	downregulated in rhabdomyosarcoma LIM protein
EC	endometrial carcinoma
EphA4	ephrin type A receptor 4
ERK	extracellular signal-regulated kinase
FADD	FAS-associated death domain
Gas1	growth arrest specific 1
GBM	glioblastoma
GSK3 $\beta$	glycogen synthase kinase-3 $\beta$
HIPK1	Homeodomain Interacting Protein Kinase 1
HAUSP	Herpes-virus-associated ubiquitin-specific protease
HGPS	Hutchinson-Gilford Progeria Syndrome
Hg	Hedgehog
HIPK2	homeodomain-interacting protein kinase 2
LFS	Li Fraumeni Syndrome
IDPs	Intrinsically Disordered Proteins
IDPRs	Intrinsically Disordered Protein Regions
IGF-1R	insulin-like growth factor 1 receptor
IR	insulin receptor
IRES	Internal ribosome entry site
IDR	Intrinsic disorder region
JNK	c-JUN N-terminal kinase
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2
MDM2	Mouse double minute 2 homolog
NBs	neuroblastomas
NCAM	neural cell adhesion molecule
NES	nuclear export signal
NF-kB	nuclear factor kappa B
NLS	nuclear localization signal
NT-3	neurotrophin-3

p38	p38 kinase
PCD	programmed cell death.
PEST	peptide sequence that is rich in proline, glutamic acid, serine, and threonine
PIG3	tumor protein p53 inducible protein 3
PKC	protein kinase C
PP2A	protein phosphatase 2A
PRD	proline-rich domain
PTM	post-translational modifications
RCC	renal cell carcinoma
REG	carboxy-terminal regulatory domain
SCCHN	squamous cell carcinoma of head and neck
SCF	Stem Cell Factor
SH3	Src homology 3-like domain
SHH	sonic hedgehog
SIRT1	silent mating type information regulation 2 homolog, sirtuin
SKPs	skin-derived precursor cells
TAD	transactivation domain
TrkA	tropomyosin receptor kinase A
TrkC	tropomyosin receptor kinase C
TUCAN	tumor-up-regulated CARD-containing antagonist of caspase nine
UNC5Hs	uncoordinated 5 homologs
USC	uterine squamous cell carcinoma
XIAP	X-linked inhibitor of apoptosis

## **I. Introduction**

# **1. Apoptosis**

## **1.1 Overview of cell death**

Genomic instability is a characteristic of most cancers. In hereditary cancers, genomic instability results from mutations in DNA repair genes and drives cancer development. In precancerous lesions that typically retain wild-type (WT) p53 function, the oncogene-induced DNA damage elicits p53-dependent apoptosis and/or senescence, which limits growth of the lesion. Activation of DNA damage checkpoint pathway can serve to remove potential DNA-damaged cells by apoptosis induction to block carcinogenesis. It has been proved that apoptotic signals help to protect genomic integrity (Kerr et al., 1972; Negrini et al., 2010).

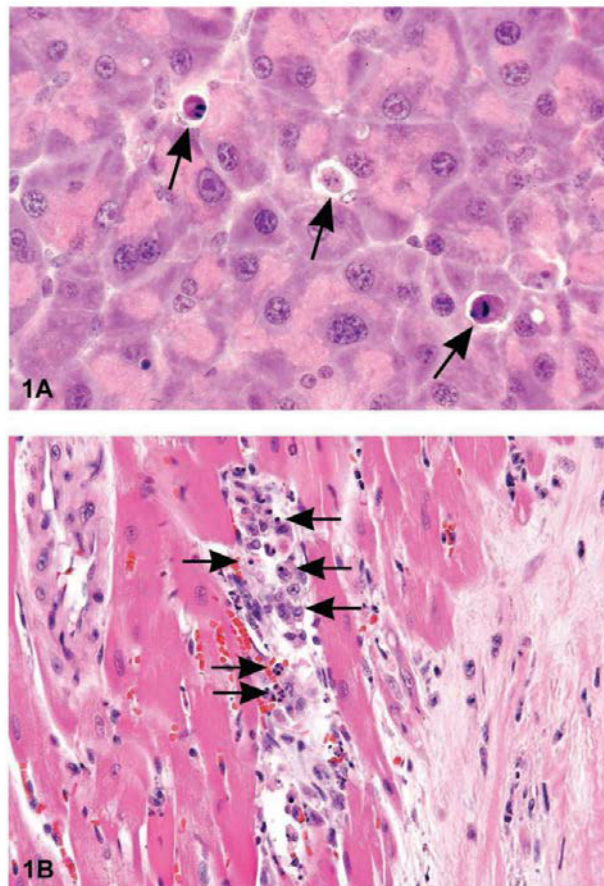
Apoptosis is a form of programmed cell death that occurs in multicellular organism. Biological events lead to characteristic cell changes morphology and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay (Elmore, 2007). Apoptosis is considered an important component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death.

Normally, apoptosis occurs during development and aging, and as a homeostatic mechanism to maintain cell populations in tissues. It also occurs as a defense mechanism, for example, in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). But inappropriate apoptosis (too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Elmore, 2007).

Though there are a number of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will die in response to the same

stimulus. Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a p53-dependent pathway. Some hormones, for example corticosteroids, results apoptotic death in some thymocytes, however other cells are unaffected or even stimulated.

The most characteristic feature of apoptosis is pyknosis, which is induced by chromatin condensation. During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy, with cell shrinkage, the cells are becoming smaller in size, the cytoplasm is dense and the organelles are more tightly packed. On histologic examination with hematoxylin and eosin stain, apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments (Figure 1) (Elmore, 2007).



**Figure 1.** Figure 1A is a photomicrograph of a section of exocrine pancreas from a B6C3F1 mouse. The arrows indicate apoptotic cells that are shrunken with condensed cytoplasm. The nuclei are pyknotic and fragmented. Figure 1B is an image of myocardium from a 14 week-old rat treated with ephedrine (25 mg/kg) and caffeine (30 mg/kg). Within the interstitial space, there are apoptotic cells with condensed cytoplasm, condensed and hyperchromatic chromatin and fragmented nuclei (long arrows). Admixed with the apoptotic bodies are macrophages, some with engulfed apoptotic bodies (arrowheads) (Elmore, 2007).



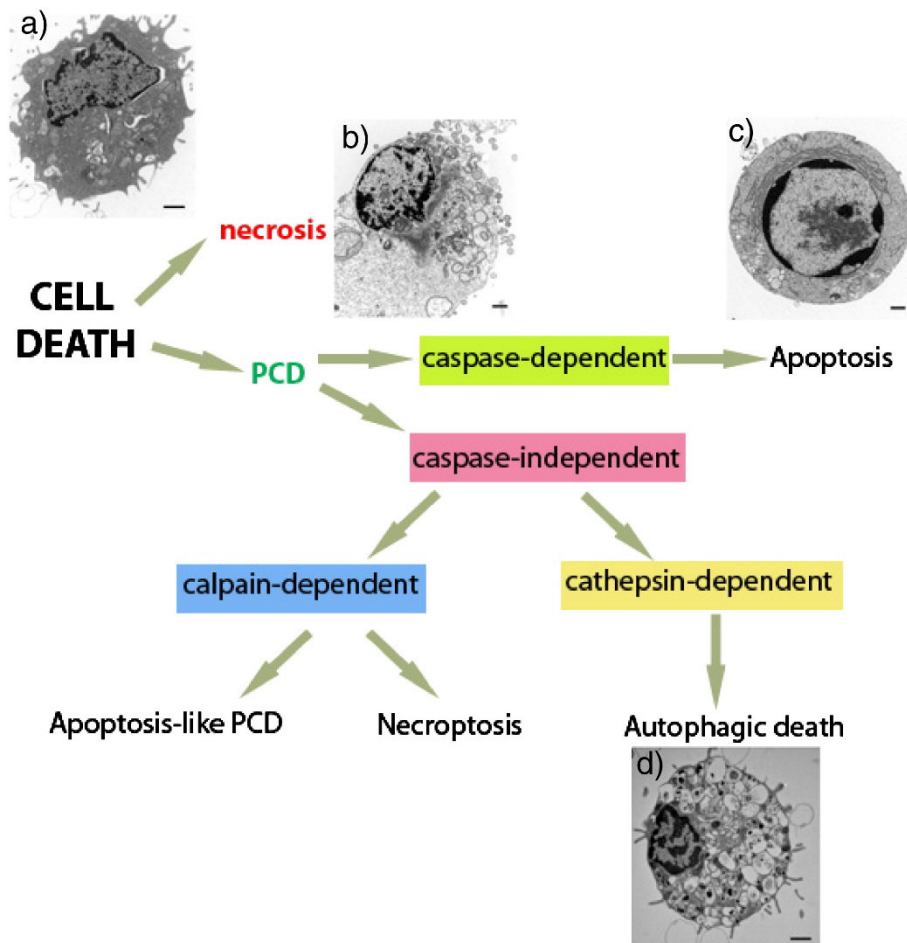
Another mode of cell death is necrosis, that, unlike apoptosis, is an alternative uncontrolled form of cell death that is induced by external injury, such as hypoxia or inflammation (Elmore, 2007). Necrosis is alternative to apoptotic cell death, and it is considered a toxic process where cell is a passive victim and follows an energy-independent mode of death. This process often involves upregulation of various pro-inflammatory proteins and compounds, such as nuclear factor nuclear factor kappa B (NF- $\kappa$ B), resulting in the rupture of the cell membrane causing spillage of the cell contents into surrounding areas, resulting in a cascade of inflammation and tissue damage (D'Arcy, 2019). In contrast to apoptosis, necrosis is an energy independent form of cell death, where the cell is damaged so severely by a sudden shock (radiation, heat, chemicals, hypoxia, etc.) that it is unable to function. The cell usually responds by swelling as it fails to maintain homoeostasis with its environment (Table 1). Necrosis is usually observed as an endpoint state in cell culture by the presence of cellular fragments in the media, what is described in many cases in a cell culture setting as necrosis is often simply the remains of late apoptotic cells, the apoptotic bodies of which have lost integrity (Nikoletopoulou et al., 2013). Since necrosis refers to the degradative processes that occur after cell death, it is considered by some to be an inappropriate term to describe a mechanism of cell death (Elmore, 2007).

Apoptosis	Necrosis
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

**Table 1** Comparison of morphological features of apoptosis and necrosis (Elmore, 2007).

Autophagy is another cell death process where cellular components such as macromolecules or even whole organelles are sequestered into lysosomes for degradation (Mizushima et al., 2008; Shintani and Klionsky, 2004). The lysosomes are then able to digest these substrates, the components of which can either be recycled to create new cellular structures and/or organelles or alternatively can be further processed and used as a source of energy. Autophagy can be initiated by a variety of stressors, most notably by nutrient deprivation (caloric restriction) or can result from signals present during cellular differentiation and embryogenesis and on the surface of damaged organelles (Mizushima et al., 2008). Recently, accumulating evidence reveals that autophagy and apoptosis can cooperate, antagonize or assist each other, thus influencing differentially the fate of the cell (Nikoletopoulou et al., 2013).

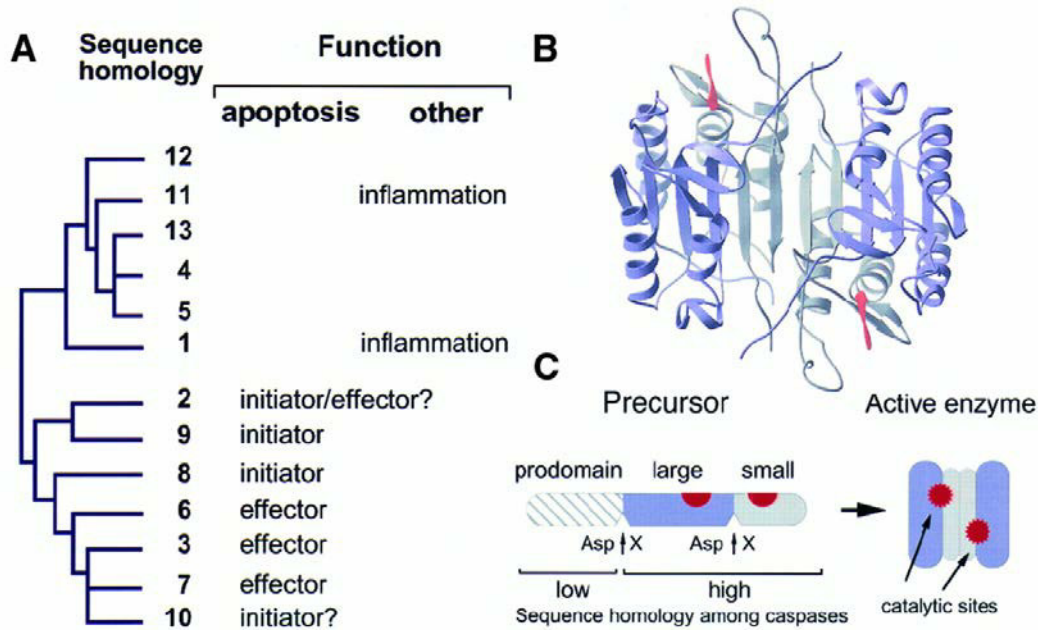
In summary, cell death may result from a number of distinct and highly regulated energy-dependent processes (apoptosis and autophagy) or from energy independent processes (necrosis). These distinct processes can be distinguished from one another based on their morphologic and biochemical behavior. Cell death can be separated into primarily non-inflammatory and pro-inflammatory categories (Figure 2). Among them, apoptosis is generally non-inflammatory and results in the orderly removal of damaged cells from tissues without inducing collateral damage to surrounding cells (Elmore, 2007).



**Fig 2.** Types of cell death and their morphological hallmarks. Diagrammatic classification of different types of cell death. PCD: programmed cell death. Morphological features of a) a healthy cell, b) a necrotic cell, c) an apoptotic cell and d) an autophagic cell (Nikoletopoulou et al., 2013).

## 1.2 Caspases

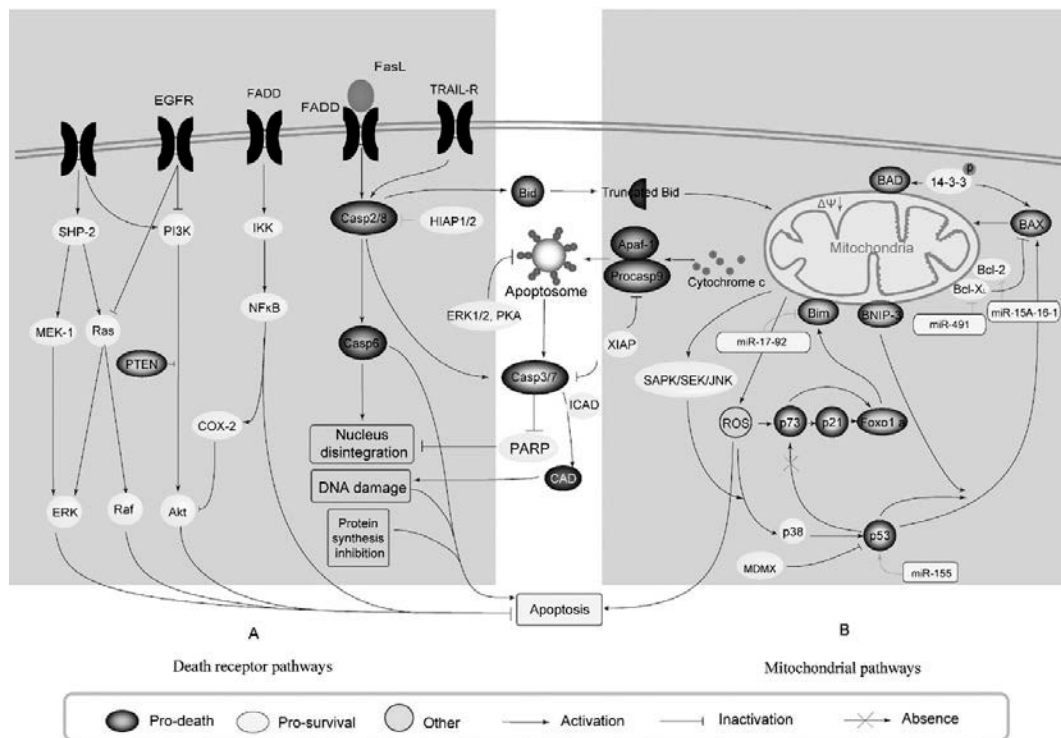
Apoptosis is an evolutionarily conserved form of cell suicide, requiring specialized machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases (cysteine-aspartic proteases). Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Once caspases are activated, there seems to be an irreversible commitment towards cell death. To date, scientists identified and categorized into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5) (Figure 3). These caspases participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell (Thornberry and Lazebnik, 1998).



**Figure 3** Proposed caspase functions and structure. **(A)** Caspases have been found in organisms ranging from *C. elegans* to humans. The 13 identified mammalian caspases (named caspase 1 to caspase 13) have distinct roles in apoptosis and inflammation. The family includes two murine homologs (11 and 12) that have no known human counterparts. In apoptosis, caspases are directly responsible for proteolytic cleavages that lead to cell disassembly (effectors) and are also involved in upstream regulatory events (initiators). The functions of caspases have been tentatively assigned based on phenotypes of knockout animals, studies of enzyme specificity, and the results of numerous other biochemical studies. **(B)** Shown is the crystal structure of caspase 3 in complex with a tetrapeptide aldehyde inhibitor (red). The active enzyme is composed of a large (~20 kDa, lavender) and small (~10 kDa, gray) subunit, each of which contributes amino acids to the active site. In the two crystal structures that are available, two heterodimers associate to form a tetramer. **(C)** In common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation. The NH<sub>2</sub>-terminal domain, which is highly variable in length (23 to 216 amino acids) and sequence, is involved in regulation of these enzymes (Thornberry and Lazebnik, 1998).

### 1.3 Apoptotic pathways

An overview of apoptotic pathways as well as the related genes are revealed in Figure 6. The mechanisms of apoptosis are complex, involving an energy dependent cascade of molecular events. To date, research indicates that there are two main apoptotic pathways: the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway) (Elmore, 2007; Ouyang et al., 2012).



**Figure 4.** Apoptotic signaling pathways as well as the related genes (Ouyang et al., 2012)

### 1.3.1 The extrinsic pathway

The extrinsic pathway is triggered by binding of Fas and other similar death receptors. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. The sequence of events that define the extrinsic apoptosis are best characterized with the FasL/FasR and TNF- $\alpha$ /TNFR1 models. In these two models, the starting of the death signal requires the death ligand binding to the receptor. Once 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 (Grimm et al., 1996; Hsu et al., 1995; Wajant, 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a 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.

### **1.3.2 The intrinsic pathway**

The intrinsic pathway leads to apoptosis under the control of mitochondrial enzymes. These proteins govern mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic, they can determine if the cell commits to apoptosis or aborts the process. Intrinsic pathway is always initiated by a range of exogenous and endogenous stimuli, such as DNA damage, ischemia, and oxidative stress (Elmore, 2007). Stimuli cause changes in the mitochondrial membrane that results in an opening of the mitochondrial permeability transition pore, mitochondrial outer membrane permeabilization (MOMP) and release of other components like cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai and Jones, 1999; Du et al., 2000; Odinkova et al., 2009). Cytochrome c binds and activates Apaf-1 as well as procaspase 9, forming apoptosome (Chinnaiyan, 1999; Hill et al., 2004). Then procaspase 9 leads to caspase-9 activation. Active caspase 9 trigger further caspase 3 activation, bringing cells to execution phase of apoptosis. Factor like XIAP is able to inhibit the activity of apoptosome, while Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting XIAP (Schimmer, 2004; van Loo et al., 2002).

### **1.3.3 The execution pathway**

Both extrinsic and intrinsic pathways end at the point of the execution phase, which is considered as the final step of apoptosis. Execution phase starts with the activation of the execution caspases, which then activate both cytoplasmic endonuclease that degrades nuclear material and proteases that degrade the nuclear and cytoskeletal proteins (Li et al., 2001). “Executioner” caspases (caspase 3, caspase 6, and caspase 7) cleave 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.

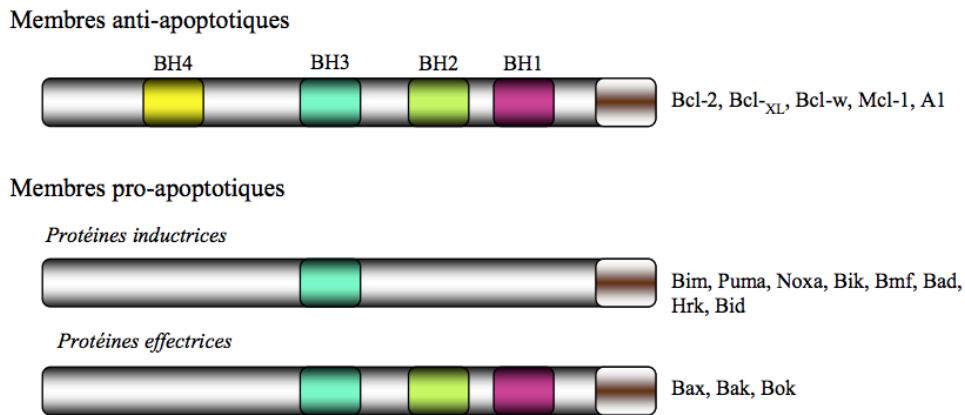
## **1.4 Key regulatory factors of apoptosis in cancer**

Cancer, a complex genetic disease resulting from mutation of oncogenes or tumor suppressor genes, can be developed due to alteration of signaling pathways; it has been well known to have numerous links to programmed cell death (PCD). Apoptosis is the major type of cell death that occurs when DNA damage is irreparable (Llambi and Green, 2011; Ouyang et al., 2012). Accumulating evidence has shown that abnormal expression of some key regulatory factors may lead to cancer, indicating the intricate relationships between apoptosis and cancer (Ouyang et al., 2012).

### **1.4.1 Bcl-2 family**

The Bcl-2 family includes key regulators of apoptosis and the molecule is overexpressed in many types of cancer cell (Llambi and Green, 2011). The whole Bcl-2 family consists of more than 20 members with either pro-apoptotic or anti-apoptotic functions and is divided into three groups based on the presence of conserved Bcl-2 homology (BH) regions. The multi-region anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1 contain all four BH regions. The pro-apoptotic proteins are divided into two groups. The multi-domain pro-apoptotic proteins, Bax, Bak and Bok were conventionally thought to share BH 1-3 regions, whereas the BH3-only proteins were proposed to share homology in the BH3 region only. Members of this diverse subset include Bad, Bim, Bid, Noxa, Puma, Bik/Blk, Bmf, Hrk/DP5, Beclin-1 and Mule (Engel and Henshall, 2009) (Figure 5). All BH3-only molecules require multi-domain BH3 proteins (Bax and Bak) to apply their intrinsic pro-apoptotic activities (Shamas-Din et al., 2011). These leads to release of cytochrome c and secondly mitochondria derived activator of caspases; the outer mitochondrial membrane becomes permeable in response to apoptotic stimuli, while cytochrome c can interact with Apaf-1 (apoptotic peptidase activating factor-1) once released into the cytosol, leading to activation of caspase

9. Activated caspase 9 activates caspase 3, subsequently activating the downstream caspase cascade finally generating apoptosis (Wen et al., 2012).



**Figure 5.** Structure of Bcl-2 family members (Guenebeaud, 2010).

Bid is the link between the extrinsic and the intrinsic apoptotic pathway. Bid connects activation of the extrinsic death receptor pathway to activation of the mitochondrial-disruption processes associated with the intrinsic pathway. Activation of Bid involves cleavage of cytoplasmic Bid by caspase 8 to expose a new N-terminal glycine residue, which undergoes post-translational myristoylation. Myristoylated Bid translocates to the mitochondria, inserts into the membrane and simultaneously activates BAX and BAK to initiate mitochondrial events leading to apoptosome (explained in the next paragraph) formation (Haupt et al., 2003).

### 1.4.2 Apaf-1 (apoptotic peptidase activating factor-1) and cytochrome c

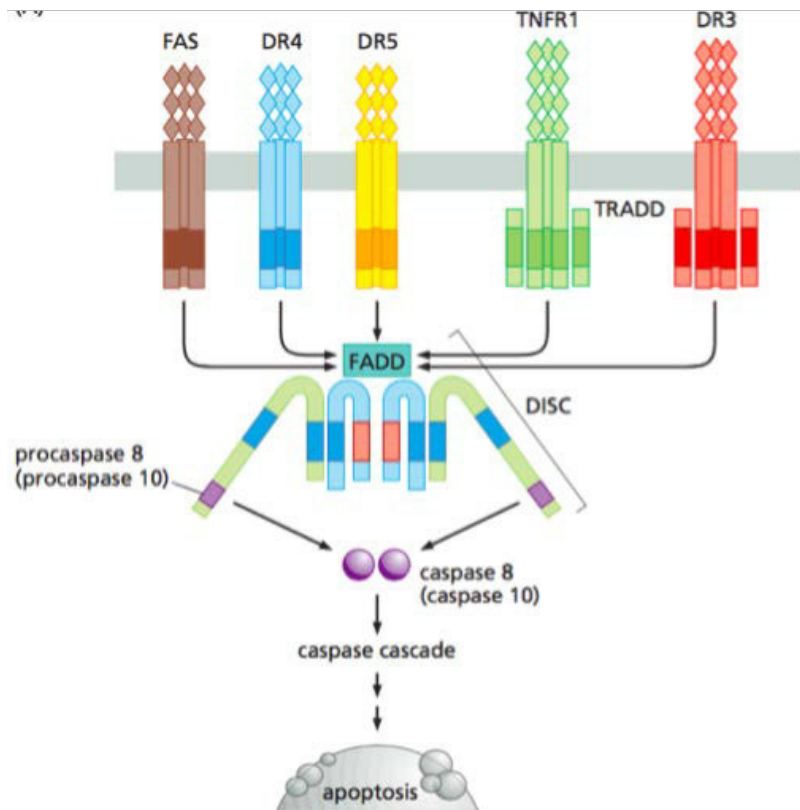
Caspase 9 is one of the most representative initiators of mitochondrial apoptosis. Facing apoptotic stimuli, for example agents that induce DNA damage or inhibit DNA repair, pro-apoptotic BH3-only proteins Bim, Bid and Bad can be activated, and promoting oligomerization of p53 effector Bax/Bak, permeabilization of the mitochondrial outer membrane, then release of factors from the intermembrane space (Li et al., 1997; Zou et al., 1997), pro-survival members Bcl-2, Bcl-XL, and Mcl-1 counteract this effect (Kelly and Strasser, 2011). It has been observed that caspase 2, containing a caspase recruitment domain (CARD) and multi-protein complex, may act upstream of mitochondrial permeabilization by cleaving and



activating Bid, playing an important role when DNA damage has induced apoptosis (Ghavami et al., 2009). In the cytoplasm, cytochrome c binding to Apaf-1 in the presence of dATP or ATP, promotes self-oligomerization of Apaf-1 (Scaffidi et al., 1998). It is important to observe that both Apaf-1 and caspase-9 contain the protein interaction motif CARD, while Apaf-1 strongly binds caspase 9 through CARD–CARD interactions, leading to formation of a cytoplasmic feature called the apoptosome, then dimerization-induced activation of caspase 9 (Chen and Wang, 2002). Apaf-1 recruit some executioners caspases 3 and caspase 7 as soon as the core complex is formed and caspase 9 is activated. These executioners cleave the targeted key regulatory molecule and structural proteins, for proteolysis to bring about apoptotic cell death (Bratton et al., 2001).

### **1.4.3 Death receptor family**

The death receptor (DR) family which includes tumor necrosis factor receptor TNF-R1, Fas, DR3, TRAIL-R1/2 (DR4/5) and DR6 can initiate the extrinsic pathway leading to apoptosis (Sayers, 2011) (Figure 6). Similarity between these family members mainly relies on the cytoplasmic regions of receptors, namely the death domains (DD), which when bound to their appropriate ligands recruit Fas-associated death domain (FADD). When pro-caspase 8 becomes hydrolysed into active caspase 8, the recruited adaptor protein containing death-effector domain (DED), can interact with DED of pro-caspases 10, thus aggregating as a DISC (Mannick et al., 1999).



**Figure 6.** Death receptors.

Five families of “death receptor” proteins are displayed on the surfaces of various types of mammalian cells. The extrinsic pathway requires the ligation of death receptors by death ligands, which results in the assembly of adaptor molecules and pro-caspase-8 activation. Again, executioner caspases 3 and 7 are then activated by caspase-8, finally induces cell apoptosis (Chipuk and Green, 2006; Weinberg, 2013).

The ligands of these receptors are TNF- $\alpha$ , TRAIL and FASL, which are belong to tumor necrosis factor family. They share the same cytoplasmic death domain and bind to its own receptor (Table 2).

Receptor	Ligand
TNFR1	TNF, LT $\alpha$
CD95 (Fas)	CD95L(FasL)
DR3	TL1A
DR4 (TRAILR1)	Apo2L/TRAIL
DR5 (TRAILR2)	Apo2L/TRAIL

**Table 2** Death receptors and their ligands (Wilson et al., 2009)

When stimuli occur as Fas combines with Fas-L, death complex recruiting FADD and pro-caspase 8, formation of the DISC is initiated to activate caspase 8 (Bell et al., 2008; Sun, 2011).

#### **1.4.4 NF-kappaB**

NF-kappaB (nuclear factor kappa B) is a class of protein with various transcriptional regulatory functions involved in stress responses, cell proliferation, differentiation, apoptosis and tumorigenesis, and significance of IKK/NF-kB in apoptosis has been described (Ghobrial et al., 2005). NF-kappaB activation is initiated by signal-induced degradation of IκB protein, which binds NF-kappaB acting as an inhibitor, resulting in its inactivation. After degradation of IκB, NF-kappaB can acquire the opportunity to enter the cell nucleus where it can perform its transcriptional regulation function and turn on expression of certain appropriate genes to avoid apoptosis (Karin and Greten, 2005; Kuhnel et al., 2000; Nelson et al., 2004). IKK is deemed to be the main regulator of activation of the NF-kappaB signaling pathway, thus, IκB phosphorylation is suppressed by activation of IKK, bringing about inactivation of NF-kappaB pathways and indirectly promoting cell apoptosis (Karin et al., 2004; Yin et al., 1998). Activation of the IκK/ NF-kappaB signaling pathway leads to induction of target genes that can interfere with the apoptotic process (Ling et al., 2011; Song et al., 2012).

#### **1.4.5 p53**

The nuclear transcription factor p53 can govern main apoptotic signals that mitochondria receive in the intrinsic pathway of apoptosis. p53 is an important pro-apoptotic factor and tumor inhibitor, thus numerous anti-tumor drugs can exert their functions by targeting p53-related signaling pathways (Kastan et al., 1991). p53 mainly promotes apoptotic cell death by activating a number of positive regulators of apoptosis such as DR-5 and Bax (Chipuk and Green, 2006). We will deepen apoptosis induced by p53 after, in p53 chapter.

## **1.4.6 Inhibition of apoptosis protein (IAPs)**

The inhibitor of apoptosis (IAPs) are a group of structurally and functionally similar proteins that regulate apoptosis, cytokinesis, and signal transduction. IAPs are defined by the presence of the baculovirus IAP repeat (BIR) domain(s), an approximately 70-residue-large zinc-binding domain that mediates protein–protein interactions. IAPs, of which there are eight in humans, namely X-chromosome–linked IAP (XIAP, also known as hILP, MIHA, and BIRC4), cellular IAP 1 (c-IAP1, also known as HIAP2, MIHB, and BIRC2), c-IAP2 (also known as HIAP1, MIHC, and BIRC3), neuronal apoptosis inhibitory protein (also known as BIRC1), survivin (also known as TIAP and BIRC5), Apollon (also known as Bruce and BIRC6), melanoma IAP (ML-IAP, also known as KIAP, livin, and BIRC7), and IAP-like protein 2 (also known as BIRC8) (Vucic and Fairbrother, 2007).

IAPs are a family of proteins with various biological functions including regulation of innate immunity and inflammation, cell proliferation, cell migration and apoptosis (Berthelet and Dubrez, 2013). In mammals, IAPs regulate apoptosis through controlling caspase activity and caspase-activating platform formation. XIAP and cellular cIAPs appeared to be important determinants of the response of cells to endogenous or exogenous cellular injuries, converting the survival signal into a cell death-inducing signal (Berthelet and Dubrez, 2013; Silke and Meier, 2013).

## **1.5 Major ways of regulating apoptosis in cancer**

### **1.5.1 Bcl-2 family protein regulation**

As mentioned, Bcl-2 family is overexpressed in many types of cancer cell (Llambi and Green, 2011). While reduced Bcl-2 expression may promote apoptotic responses to anticancer drugs, increased expression of Bcl-2 leads to resistance to chemotherapeutic drugs and radiation therapy (Engel and Henshall, 2009). 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. It can be due to an overexpression of one or more anti-apoptotic proteins or an down

regulating expression of one or more pro-apoptotic proteins or a combination of both. Evidence showing that overexpression of Bcl-2 protected prostate cancer cell from apoptosis (Raffo et al., 1995), and overexpression of Bcl-2 also led to inhibition of TRAIL-induced apoptosis in neuroblastoma, glioblastoma or breast carcinoma cell lines (Fulda et al., 2002).

### **1.5.2 Inactivation of p53**

As mentioned, p53 is an important pro-apoptotic factor and tumor inhibitor, defects in the p53 tumor suppressor have been linked to more than 50% of human cancers (Ozaki and Nakagawara, 2011). Mutant p53 acts as the dominant-negative inhibitor toward wild-type p53. Indeed, mutant p53 has an oncogenic potential. In some cases, malignant cancer cells bearing p53 mutations display a chemoresistant phenotype (Ozaki and Nakagawara, 2011), and numerous anti-tumor drugs can exert their functions by targeting p53-related or p53 mutants-related signaling pathways (Kastan et al., 1991).

### **1.5.3 Upregulation of inhibitor of apoptosis proteins (IAPs)**

Dysregulation IAP expression has been reported in many cancers. For example, IAP family was discovered abnormally expressed in pancreatic cancer cell and this abnormal expression was responsible for resistance to chemotherapy (Lopes et al., 2007). Another IAP family member, BIRC7 was proved highly expressed in melanoma and lymphoma (Ashhab et al., 2001; Lopes et al., 2007). Moreover, Apollon (BIRC6) was demonstrated upregulated in gliomas and was responsible for cisplatin and camptothecin resistance (Chen et al., 1999).

### **1.5.4 Reduced caspase activity**

As discussed before, caspases is 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. Study showing that 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., 2003). Researchers also demonstrated caspases-3 mRNA levels in total RNA samples from breast, ovarian, and cervical tumors were either undetectable (breast and cervical) or substantially decreased (ovarian) (Devarajan et al., 2002).

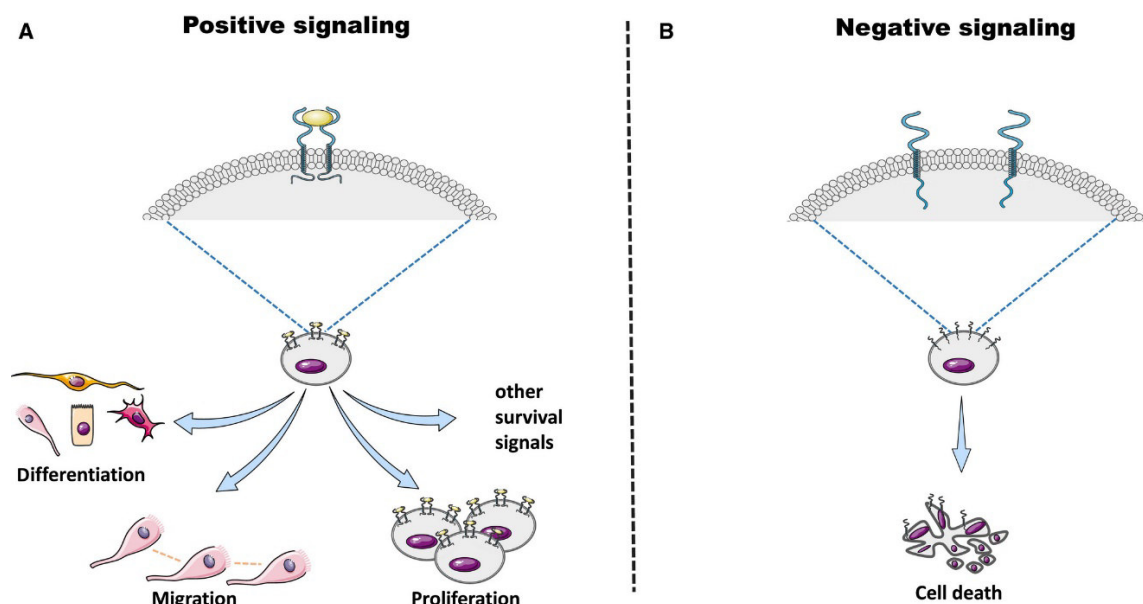
### **1.5.5 Impaired death signaling**

Death receptors are key players in the extrinsic pathway of apoptosis. Abnormalities in death signaling pathways can lead to evasion of extrinsic pathway of apoptosis. Such abnormalities include downregulation of the receptors and impairment of receptor function, as well as the reduced level in the death signals, all of which contribute to impaired signaling and a reduction of apoptosis. For example, downregulation of receptor expression has been indicated in some studies as a mechanism of acquired drug resistance. Reduced Fas receptor expression was found to play a role in treatment-resistance leukemia (Ramp et al., 2000) or neuroblastoma (Fulda et al., 1998). Moreover, reduced expression of death receptors and abnormal expression of decoy protein of these receptors, which bind to death ligand without containing death domain, have been reported to play a role in the evasion of death signaling pathway in various cancer (Fulda, 2010).

## 2. Dependence receptors

### 2.1 Dependence receptor concept

The classical membrane receptors are considered as inactive unless they bound to their ligand(s). However, recent years increasing studies demonstrate that some receptors, in addition to their ‘positive’ signaling when their ligand is present, transduce a ‘negative’ signal that induces apoptosis in the absence of their ligand. Cells expressing these receptors become dependent on the presence of ligand to survive. Thus, these receptors are named ‘dependence receptors’ (DR) (Figure 7).



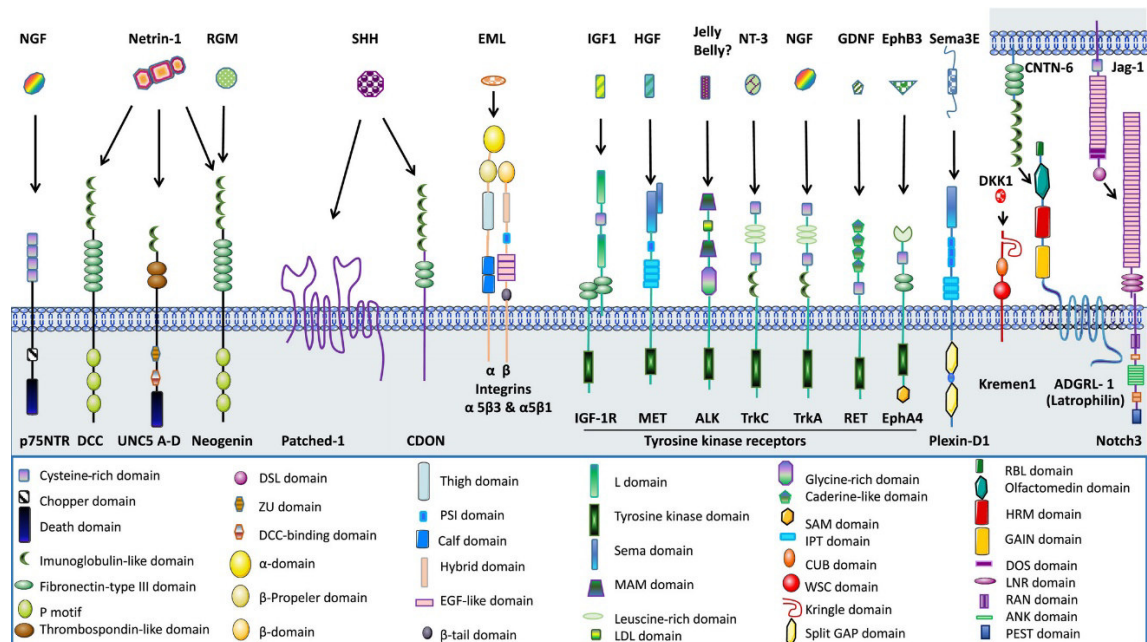
**Figure 7.** The dependence receptor paradigm.

(A) Positive signaling. When ligand binds with dependence receptors, the receptors could trigger various survival signals such as migration, differentiation and proliferation. (B) Negative signaling. However, In the absence of ligand, the dependence receptor induces cell death in an active manner (Negulescu and Mehlen, 2018).

### 2.2 Dependence receptor family

At present, the DR family comprises around twenty members whose homology is limited to their functional duality (Figure 8). The family members contain Netrin-1 receptors, deleted in colorectal carcinoma (DCC) (Mehlen et al., 1998) and uncoordinated 5 homologs (UNC5Hs, UNC5A, B, C, D) (Arakawa, 2004; Llambi et al., 2001), Neogenin receptor (Matsunaga et al., 2004), p75 neurotrophin receptor (p75<sup>NTR</sup>) (Rabizadeh et al.,

1993), receptors to the morphogen sonic hedgehog (SHH), patched-1 (PTCH-1/ Ptc) and cell-adhesion molecule- related/downregulated by oncogene (CDON) (Delloye-Bourgeois et al., 2013; Thibert et al., 2003), Semaphorin-3E receptor Plexin-D1 (Luchino et al., 2013), some integrins (Stupack et al., 2001), insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) (Boucher et al., 2010), and anaplastic lymphoma kinase (ALK) (Mourali et al., 2006), Moreover, several receptors with tyrosine kinase activity were added to DR family, such as rearranged during transfection (RET) (Bordeaux et al., 2000), tropomyosin receptor kinase C (TrkC) (Tauszig-Delamasure et al., 2007), ephrin type A receptor 4 (EphA4) (Furne et al., 2009), MET (Tulasne et al., 2004), and more recently c-Kit (Wang et al., 2018). In addition, Notch3 (Lin et al., 2017) and Kremen-1 (Causeret et al., 2016) were also newly discovered.



**Figure 8.** Dependence receptor family. Structure of the currently known dependence receptors and their ligands (Negulescu and Mehlen, 2018).

### 2.3 Mechanism of dependence receptors induce apoptosis

So far, all the DRs trigger cell death by apoptosis. Several studies have shown that they can localize in lipid rafts for the transduction of both the positive and negative signaling for cell (Furne et al., 2006; Goldschneider and Mehlen, 2010; Maisse et al., 2008). One of the molecular hallmarks of apoptosis is the activation of caspases. During apoptosis, caspases (a family of cysteine-dependent aspartate-directed proteases) can cleave a wide



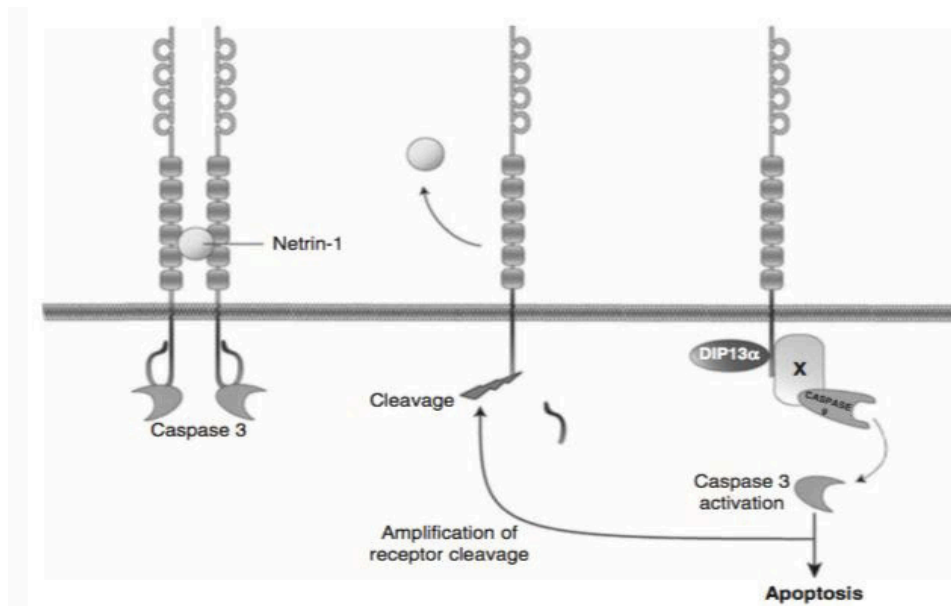
range of substrates, inactivating survival and activating pro-apoptotic pathway (Negulescu and Mehlen, 2018). Except for the fact that they induce apoptosis in the absence of their ligand, the other most common characteristic is that DR are cleaved by caspases. Receptor cleavage is important for apoptotic function, as mutation of the cleavage site abolishes cell death induction. DCC, neogenin, c-Kit, Ptc, ALK, EphA4 are cleaved once, roughly in the middle of their intracellular domain, UNC5H cleavage site is very close to the plasma membrane, and RET, TrkC and MET have two cleavage sites. Interestingly in mammals, caspase cleavage sites of DRs seem to be conserved, but variable in other vertebrates, and never in lower organisms (Table 3). Above results showing that the appearance as a caspase substrate, and therefore the mediation of the dependence state, is a relatively late event in evolution of these proteins. It allows greater plasticity of the mammalian nervous system compared with those of invertebrates and the necessity for more complex and higher lifespan mammals to develop antitumor mechanisms (Goldschneider and Mehlen, 2010).

	Homo sapiens	Pan troglodytes	Bos taurus	Canis familiaris	Mus musculus	Rattus norvegicus	Gallus gallus	Xenopus tropicalis	Xenopus laevis	Danio rerio
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 DYLD	VSVD DYLD	VSVD DYLD	VSVD DYLD	VPVD DYLD	VSVD DYLD	VSVD DYLD	NA	MSVD DYLD	VAID DYLD
TrkC	SSLD ILVD	SSLD ILVD	SSLD ILVD	SSLD ILVD	SSLD ILVD	SSLD ILVD	SSLD ILVD	NA	NA	NA
EphA4	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEDD	LEED
ALK	DELD	DELD	DELD	DELD	DELD	DELD	DELD	DEMD	NA	DELD
MET	ESVD DNAD	ESVD DNAD	ESVD No	ESVD DNID	ESVD DNID	ESVD DNID	ESVD DNTD	ESVD No	ESVD No	ESVD SNLD?

**Table 3** Conservation of caspase cleavage sites of DR among species (Goldschneider and Mehlen, 2010).

DRs share the property of being caspase amplifiers, most of them fail to induce apoptosis in the presence of general caspase inhibitors such as zVAD.fmk or the baculovirus protein p35 (Mehlen and Thibert, 2004). Another common feature of these receptors is the presence of a domain required for the pro-apoptotic activity of these unligated receptors. These domains, called addition/dependence domains (ADDs), are required and often sufficient for cell death induction (Bredesen et al., 1998). Such specific domain has been mapped to the intracellular domain of DCC, just upstream of the caspase cleavage site (D1290), from amino acid 1243-1264 (Mehlen et al., 1998). The deletion of this domain is sufficient to eradicate the pro-apoptotic activity of DCC. Except for p75<sup>NTR</sup> and integrins, caspase cleavage is thought to be responsible for unmasking the ADD. In most cases, ADD is borne by the remaining membrane-anchored fragment. However, in the UNC5H, TrkC, RET, and MET receptors, it is the cytosolic-generated fragment that is pro-apoptotic. ADD domains are unique regions that lack of structural homology with other known functional protein domains (Negulescu and Mehlen, 2018).

After ADD release, caspase amplification become more or less direct, depending on receptors. In some cases, ADD recruits caspase-activating. For example, in the absence of Netrin-1, the receptor DCC recruits and activates caspase 9, 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. DCC does not interact directly with caspase 9, but it may recruit one or more adaptor proteins. 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 (Goldschneider and Mehlen, 2010) (Figure 9).

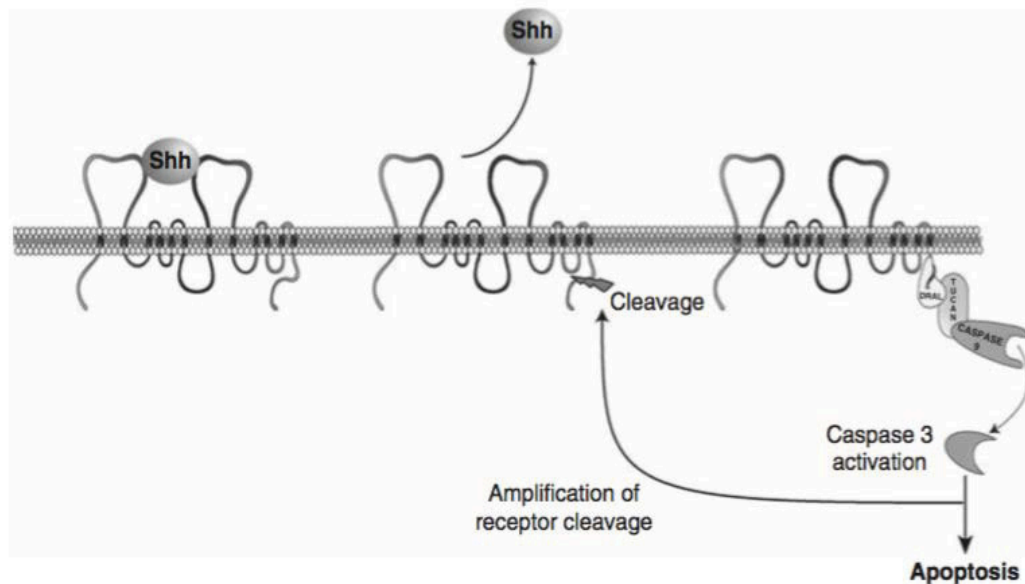


**Figure 9.** Model of cell death induction by DCC.

In the presence of netrin-1, DCC is dimerized and interacts with procaspase 3. However, in the absence of ligand, DCC becomes a monomer and is cleaved. Cleavage leads to the exposure of ADD and to its direct interaction with apoptotic partners, for example DIP13a or to indirect interaction with caspase 9. Consequently, these interactions result in activation of caspase 9, which in turn activates caspase 3 (Goldschneider and Mehlen, 2010).

The presence of a caspase-activating complex recruited to a DR has been demonstrated for Patched (Ptc) receptor. Ptc is able to interact, through its ADD, with DRAL/FHL, only in the absence of its ligand Shh (Mille et al., 2009b). DRAL (downregulated in rhabdomyosarcoma LIM protein) 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 (tumor-up-regulated CARD-containing antagonist of caspase) (Stilo et al., 2002). Ptc–DRAL association serves as a platform for recruiting TUCAN (and/or NALP1, a protein closely related to TUCAN) and caspase 9. This then allows caspase 9 recruitment to Ptc and caspase 9 activation. The complex involving DRAL, TUCAN and caspase 9 was named dependosome, by analogy to other known caspase-activating complexes (Figure 10). The dependosome also includes the E3 ubiquitin ligase NEDD4. Evidence showing that Ptc-mediated apoptosis and Ptc-induced caspase-9 activation require NEDD4. Ptc dependence receptor was proved specifically allows the activation of caspase-9 via its ubiquitination, which occurs via the recruitment by Ptc of NEDD4 (Fombonne et al., 2012).

In any case, if such a dependosome existed and were common to DRs, the initiator recruited caspase could not always be caspase 9. Indeed, Stupack showed that integrins, trigger apoptosis through recruitment of caspase 8 (Stupack et al., 2001).



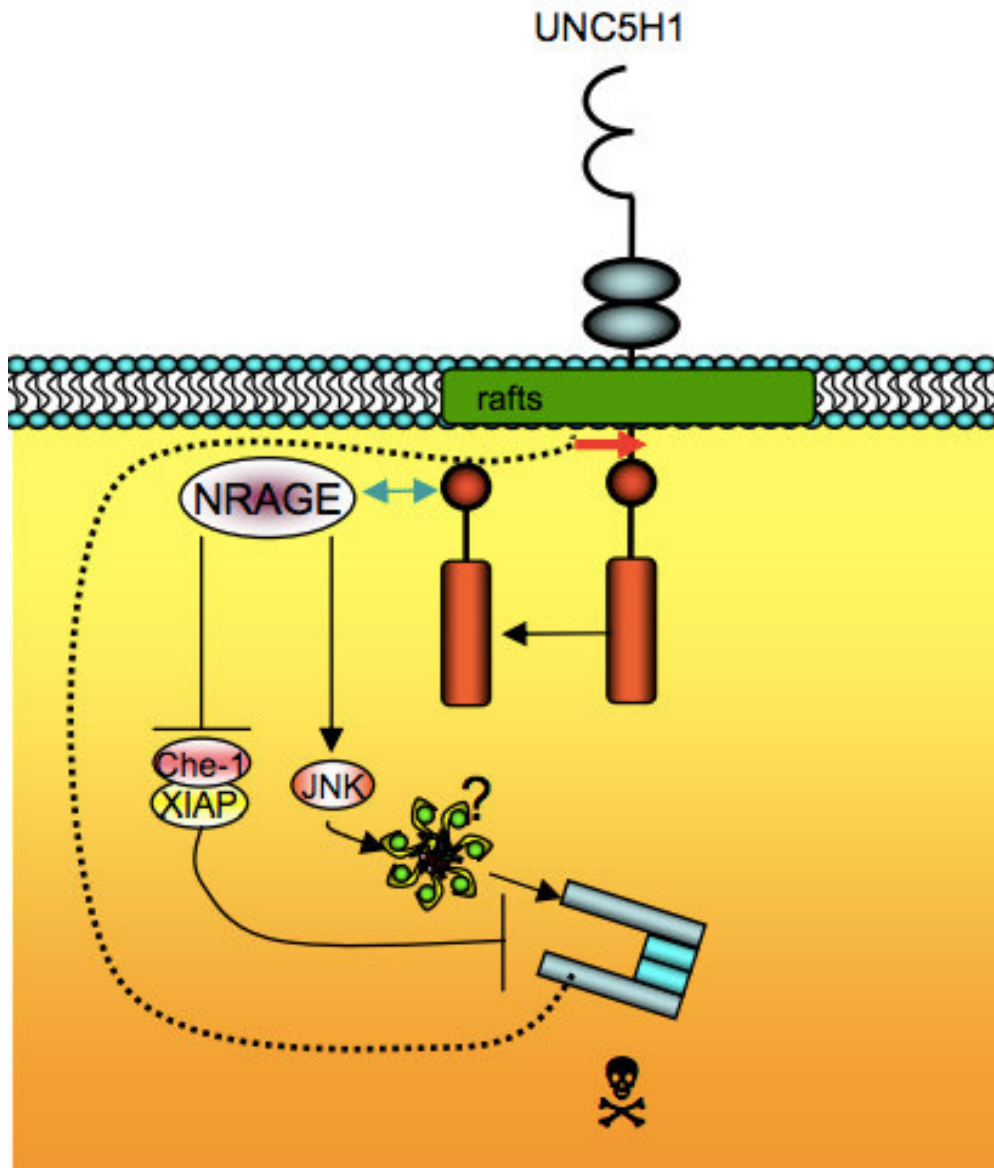
**Figure 10.** Model of cell death induction by Ptc.

Following ligand withdrawal, Ptc is cleaved by caspase (or by another activated protease), thus allowing exposure of its ADD. 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).

The formation of a caspase-activating complex does not seem to be the only mechanism used by DRs to trigger apoptosis. As an example, the mechanism of UNC5H-induced apoptosis. Despite their structural homology, members of the UNC5H family seem to mediate their apoptotic signal by interacting preferentially with distinct partners. The UNC5s contain two Igs and two thrombospondin type-I repeats in the extracellular domain. In addition, their cytoplasmic domains contain regions of homology with other proteins: 1) a ZU-5 domain homologous to Zona Occludens-1, a protein implicated in tight-junction formation (Schultz et al., 1998), and 2) a C-terminal death domain, a domain first identified as the pro-apoptotic region of tumor necrosis factor receptor-1 (Hofmann and Tschopp, 1995; Tartaglia et al., 1993). In fact, apoptotic pathways downstream of UNC5C and UNC5D have not yet been documented. Functional data are available only for UNC5A and UNC5B.

Evidence showing an interaction between UNC5A and NRAGE, and this interaction is responsible for apoptosis induced by UNC5A, after mapped the NRAGE binding domain of UNC5A to its ZU-5 domain it shows this region, in addition to an adjacent PEST sequence (peptide sequence that is rich in proline, glutamic acid, serine, and threonine), is required for UNC5A-mediated apoptosis. Moreover, UNC5A does not induce apoptosis in differentiated PC12 cells, which down-regulate NRAGE, but induces apoptosis in native PC12 cells that endogenously express high levels of NRAGE. Together, these results demonstrate a mechanism for UNC5A-mediated apoptosis that requires an interaction with the MAGE protein NRAGE (Williams et al., 2003) (Figure 11). Studies founded two mechanisms to induce apoptosis in cells: one involves NRAGE-dependent degradation of the survival protein XIAP (X-linked inhibitor of apoptosis), and the other involves NRAGE-dependent activation of the c-Jun N-terminal kinase signaling pathway and caspases (Salehi et al., 2002; Williams et al., 2003).

Apoptosis induction and NRAGE binding, specifically require the juxtamembrane region of UNC5A. The juxtamembrane region of UNC5A consists of a short PEST sequence immediately followed by a ZU-5 domain. In contrast, UNC5B, UNC5C do not contain a PEST sequence and have an insertion of 20 amino acids in length preceding the ZU-5 domain. These sequence differences may explain the differences between the UNC5Hs ability to bind NRAGE and induce apoptosis (Williams et al., 2003).

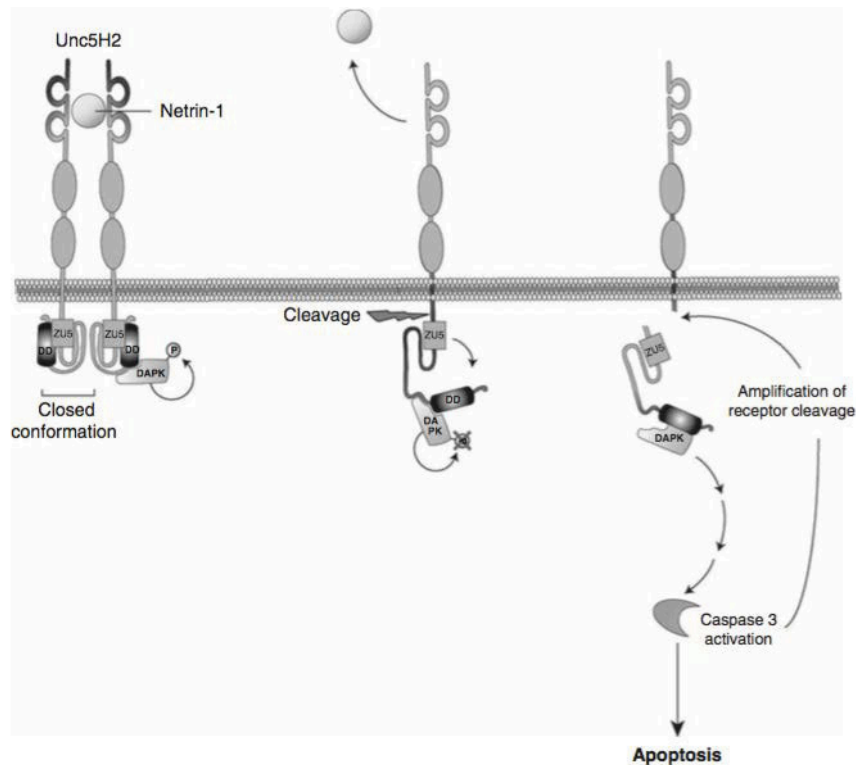


**Figure 11.** UNC5A induces apoptosis via its juxtamembrane region through an interaction with NRAGE (Williams et al., 2003).

UNC5B receptor also containing the NRAGE binding domain and PEST sequence of UNC5A, bind NRAGE and cause increased levels of apoptosis (Williams et al., 2003). Study on UNC5B revealed that receptor UNC5B triggers cell death through the activation of the serine-threonine protein kinase DAPK (Death-Associated-Protein kinase). DAPK is a crucial intracellular protein that mediates cell death induction through a wide spectrum of apoptotic and non-apoptotic signals via its serine-threonine kinase activity. UNC5B-induced apoptosis is dependent on DAPK dephosphorylation (Guenebeaud et al., 2010). By shRNA screening, structural subunit PR65 $\beta$  of holoenzyme protein phosphatase 2A (PP2A) has been identified as a protein involved in UNC5B-induced apoptosis process. UNC5B recruits a protein complex that includes PR65 $\beta$  and DAPK

and retains PP2A activity. PP2A activity is required for UNC5B-induced apoptosis, since it activates DAPK by triggering its dephosphorylation (Guenebeaud et al., 2010). DAPK activation initiates an apoptotic program via the activation of caspase 9 and caspase 3 (Goldschneider and Mehlen, 2010). Moreover, DAPK is known to be capable of autophosphorylation, which inhibits its activity by inducing a conformational change. Llambi and colleagues proposed that, in the presence of Netrin-1, DAPK is in an inactive autophosphorylated state, and it interacts with UNC5B through their non-DD-interacting regions, whereas, in the absence of netrin-1, DAPK interacts with the DD of UNC5B, which allows DAPK activation (Llambi et al., 2005) (Figure 12). However, others inhibitor also involved, for examples, it was suggested that Netrin-1 binding to UNC5B prevents apoptosis through interaction of the PP2A inhibitor CIP2A to UNC5B (Guenebeaud et al., 2010)

In the presence of netrin-1, two UNC5B receptors 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, UNC5B receptor becomes a monomer, whereas the intracellular domain undergoes both caspase (or another protease) cleavage and opening/dissociation of ZU5 and DD in parallel (Llambi et al., 2005). Relation and chronology between cleavage and opening remain unclear, but these modifications 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 (Goldschneider and Mehlen, 2010).



**Figure 12** Model of cell death induction by UNC5B receptor (Goldschneider and Mehlen, 2010).

## 2.4 Dependence receptors and cancers

### 2.4.1 Dependence receptors act as tumor suppressors in a range of cancers

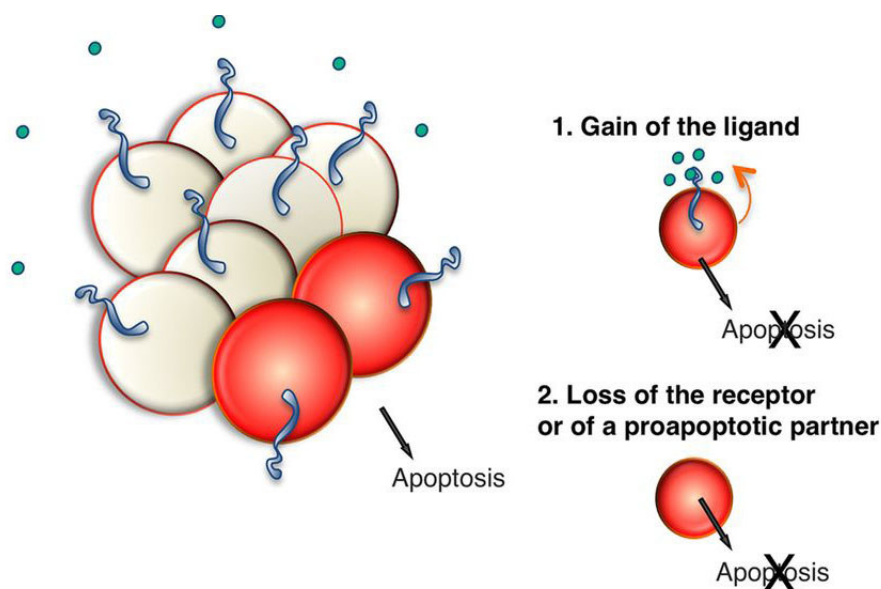
Because of the pro-apoptotic function of DRs, they play an important role in mediating tissue homeostasis during development and tumorigenesis. Dependence receptors often act as tumor suppressors in range of cancers. Indeed, the expression of these receptors represents a protective mechanism that limits tumor development through apoptosis induction in tumor cells which grow or migrate beyond the regions of ligand availability. Thus, dependence receptors have been reported to be lost or mutated in various cancer types. For example, observation that DCC expression is reduced or lost in colorectal cancer led to the proposal that DCC expression represented a constraint for disease progression and is therefore a tumor suppressor. In fact, DCC expression is lost or reduced in various cancers (Mehlen and Fearon, 2004), and loss of DCC expression is associated with poor prognosis (Shibata et al., 1996; Sun et al., 1999). Another example, Ptc is also a known tumor suppressor. Inactive mutations of Ptc, as well as loss of expression, are found in basal cell carcinoma and medulloblastoma (Wicking and



McGlenn, 2001). Ptc expression inhibits the hallmarks of cell transformation in vitro and Ptc also inhibits growth in soft agar of transformed cells. This is linked to its proapoptotic function, as growth inhibition does not occur in the presence of Shh, or of a general caspase inhibitor, or when Ptc is mutated on its caspase cleavage site (Fombonne et al., 2012; Negulescu and Mehlen, 2018; Wicking and McGlenn, 2001). There is a wide range of data supporting the role of most DRs as tumor suppressors. EphA4 is downregulated in invasive forms of breast cancers (Fox and Kandpal, 2004), in liver and kidney cancers and in metastatic melanomas (Hafner et al., 2004), moreover, a progressive decrease in p75<sup>NTR</sup> expression is described in prostate cancers (Perez et al., 1997; Pflug et al., 1992). TrkC is associated with good prognosis in several cancers (Bernet et al., 2007; Yamashiro et al., 1997).

## 2.4.2 How to avoid pro-apoptotic activity induced by DR?

To date, tumors have been reported to avoid the pro-apoptotic activity of DRs *via* three main mechanisms: gain of ligands, loss or mutation of the receptor, loss or mutations of pro-apoptotic partners of the receptors (Figure 13).



**Figure 13** The dependence receptors, new anti-tumoral sentinels.

Dependence receptors constitute a new anti-tumoral mechanism, eliminating undesirable cells in a tissue where the ligand is present in a limited amount. Two selective advantages may be acquired by tumoral cells to bypass this surveillance: 1) the autocrine production of ligand or 2) the invalidation of the receptor or of a pro-apoptotic partner (Mehlen and Tauszig-Delamasure, 2014).

## 1) Gain of ligands

DRs induce cell death in territories where the ligand is limited, a selective advantage for a tumor is to avoid the DR Damocles sword by producing the appropriate DR ligands. This may have two advantages for the tumor: the ligand may stimulate the positive signaling of these DRs that is often associated with promigratory, pro-proliferative effect. The second, and potentially more important, advantage is that it blocks DR-mediated cell death induction, allowing tumor progression and metastasis (Gibert and Mehlen, 2015). Up-regulation of ligands is usually observed in different types of cancer and their expressions could be correlated to tumorigenesis or metastasis. For example, TrkC ligand neurotrophin-3 (NT-3) is upregulated in a large fraction of aggressive human neuroblastomas (NBs) and that it blocks TrkC-induced apoptosis of human NB cell lines, consistent with the idea that TrkC is a dependence receptor (Bouzas-Rodriguez et al., 2010; Fitamant et al., 2008; Luchino et al., 2013)). UNC5B and DCC ligand Netrin-1 was highly expressed in a large fraction of metastatic breast cancers. Netrin-1 up-regulation appears as a marker of distant metastatic disease in human breast cancer. (Boussouar et al., 2020). Moreover, Netrin-1- expressing mammary metastatic tumor cell lines undergo apoptosis when netrin-1 expression is experimentally decreased or when decoy soluble receptor ectodomains are added (Fitamant et al., 2008). As we will extensively discuss below, there are several papers showing an up-regulation of Netrin-1 in several aggressive (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Paradisi et al., 2008; Paradisi et al., 2009). A recent paper reported increased expression of Semaphorin 3E (Sema3E) is correlating with metastasis in human breast cancer, Sema3E regulates tumor cell survival by suppressing apoptotic pathway triggered by the Plexin D1 dependence receptor (Luchino et al., 2013). SHH is also upregulated in many cancers in an autocrine and paracrine manner (Varnat et al., 2009). All the above information makes these ligands to be putative biomarker for prognostic and potential therapeutic targets.

## **2) Loss or mutation of the receptor**

According to the DR paradigm, a tumor, to grow independently of DR ligand presence, must silence DR-induced cell death, and the simplest way to achieve this would be to inactivate the receptor. Interestingly, several receptors like DCC, UNC5H, TrkC, and CDON, have been shown to be negatively regulated in various cancer types (Gibert and Mehlen, 2015). DCC is reported to be lost not only in colorectal cancer but also in prostate cancer, breast cancer, ovarian cancer and neuroblastoma (Mehlen and Fearon, 2004). Moreover, mutation of the caspase cleavage site of DCC is sufficient to include neoplasia in intestine and increase the tumor incidence in an adenomatous polyposis coli (APC)-mutated mouse model (Castets et al., 2011). Loss or inactivating mutations of Ptc have been observed in basal cell carcinoma and medulloblastoma (Wicking and McGlinn, 2001). In addition, the UNC5A-C are also found to be lost and behave as tumor suppressors in numerous cancers, particularly in cancer of the ovary, breast, uterus, stomach, lung and kidney and especially in the colon cancer (Shin et al., 2007; Thiebault et al., 2003). p75<sup>NTR</sup> is partially lost in the localized prostate tumor epithelium, and this loss is inversely correlated with tumor grade and total losses have been observed in metastatic prostate cancer lines (Perez et al., 1997; Pflug et al., 1992). Notch3 receptor is as well downregulated in breast cancer and this loss is associated with poor survival (Cui et al., 2013; Zhang et al., 2016).

## **3) loss or mutation of their pro-apoptotic partners**

Cancer cells can also silence or loss downstream pro-apoptotic partner of receptor to facilitate tumor progression. An example of DAPK1, a pro-apoptotic partner of the UNC5 family whose expression is lost in colon cancer, breast cancer and lung cancer via the methylation of its promoter (Grandin et al., 2016a; Grandin et al., 2016b).

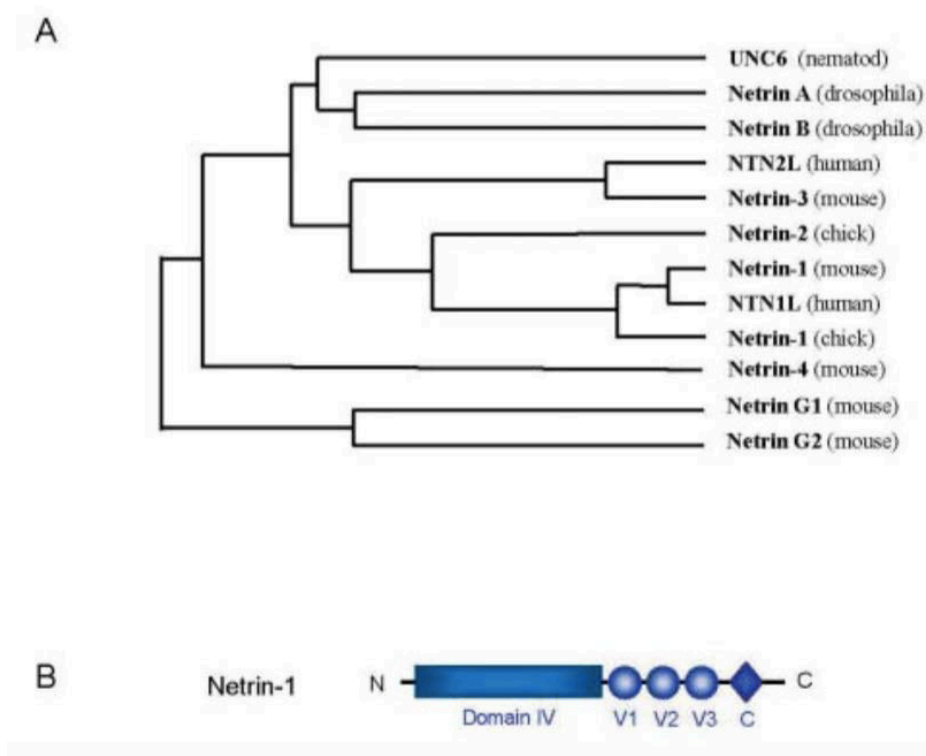
## **2.5 Netrin and its receptors DCC and UNC5Hs**

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 (Mehlen

and Llambi, 2005). Recently, more and more biological functions are described in many reporters.

### 2.5.1 Overview of Netrin

Netrin family is a wide family of conserved proteins. Several members of the netrin gene family have been identified in mammals: netrin-1, netrin-3, netrin-G1, netrin-G2 and netrin-4, also called b-netrin. Orthologs of these netrin family members have been identified in the human genome, and the human ortholog of netrin-1 is also named NTN1L. All encode ~60–80 kDa proteins composed of three domains (V, VI and C) and an amino terminal signal peptide characteristic of secreted proteins. Domains V and VI of netrins are homologous to domains V and VI of laminins, while the netrin C domain shares some sequence similarity with domains present in the complement and tissue inhibitors of metalloprotease (TIMP) protein families (Figure 14) (Mehlen and Furne, 2005).



**Figure 14.** Netrin-1 within netrins. (A) Representation of the different netrins identified so far. (B) Schematic representation of netrin-1, a laminin-related molecule (Mehlen and Furne, 2005).

Netrin-1 is one of the most well-studied members of Netrin family proteins with roles axon guidance (Deiner et al., 1997; Kennedy et al., 1994; Mehlen and Rama, 2007), axon branching (Dent et al., 2004), synaptogenesis (Flores, 2011), cell migration (Ylivinkka et al., 2016), cell survival (Mehlen and Furne, 2005), and axon regeneration (Dun and Parkinson, 2017).

## **2.5.2 Netrin-1 main receptors**

### **1) DCC**

DCC gene encodes different proteins as a result of alternative splicing (Reale et al., 1994), though all known isoforms appear to be type I transmembrane glycoproteins of 175–190 kDa with a single membrane spanning domain. The sequences present in the large extracellular domain bear strong similarity to those found in neural cell adhesion molecule (NCAM) protein family members, and include four immunoglobulin-like domains and six fibronectin type III- like motifs (Figure 15). The cytoplasmic domain of 325 amino acids shows little similarity to proteins with well-established functions. In fact, in cytoplasmic domain, three regions P1, P2 and P3 appear more specifically conserved among orthologs of DCC and are then proposed to play functional roles in DCC activity (Hong et al., 1999). DCC have a homolog in mammals-neogenin, but little is known of this protein. In humans, low expression level of DCC has been detected in many developing and adult tissues, with the highest level in the brain (Meyerhardt et al., 1997).

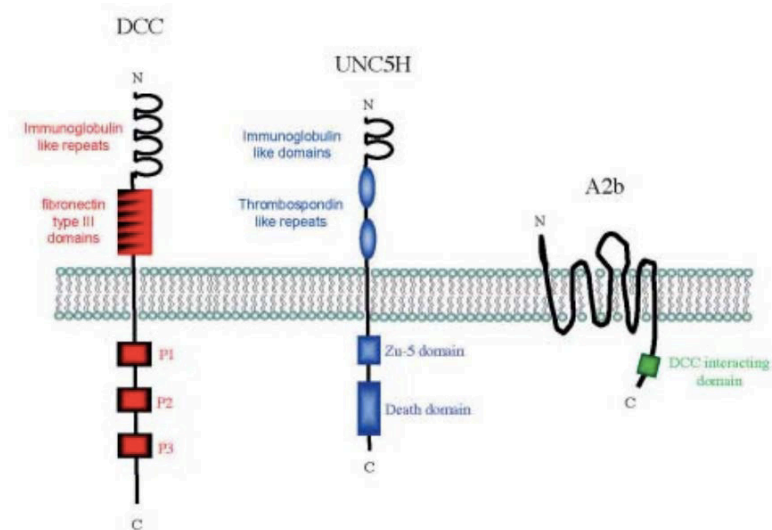
### **2) UNC5Hs**

As described before, UNC5H are type I transmembrane proteins composed of two extracellular immunoglobulin (Ig)-like domains, two thrombospondin type I domains, and an intracellular sequence that contains a ZU5 domain and a DD (Leonardo et al., 1997) (Figure 15). UNC5B functions as a repulsive netrin receptor function for the formation of the mature nervous system is now well known, in particular, their ability to control precise axon targeting (Bashaw and Klein, 2010). Moreover, UNC5B expressed by endothelial tip cells of the vascular system.

Netrin-1 causes endothelial filopodial retraction, but only when UNC5B is present. In this work, UNC5B functions as a repulsive netrin receptor in endothelial cells controlling morphogenesis of the vascular system (Lu et al., 2004).

### 3)A<sub>2b</sub>

Another type of receptor for Netrin-1 has been identified. While performing a search for intracellular partners of DCC by a two-hybrid screen, DCC actually interacted with the last intracellular domain of the G-protein-coupled receptor A<sub>2B</sub> (Corset et al., 2000) (Figure 15). A<sub>2B</sub> belongs to the family of adenosine receptors that comprises four members, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> (Fredholm et al., 2001). A<sub>2B</sub>/DCC interaction occurs mainly in the presence of Netrin-1, and Netrin-1 is able to bind directly to A<sub>2B</sub> with a dissociation constant of 22 nM independent of the presence of DCC (Corset et al., 2000).



**Figure 15.** Netrin-1 receptors. Schematic representation of netrin-1 receptors DCC, UNC5H and A<sub>2B</sub> (Mehlen and Furne, 2005).

### 2.5.3 Apoptotic signaling of UNC5B in absence of Netrin-1

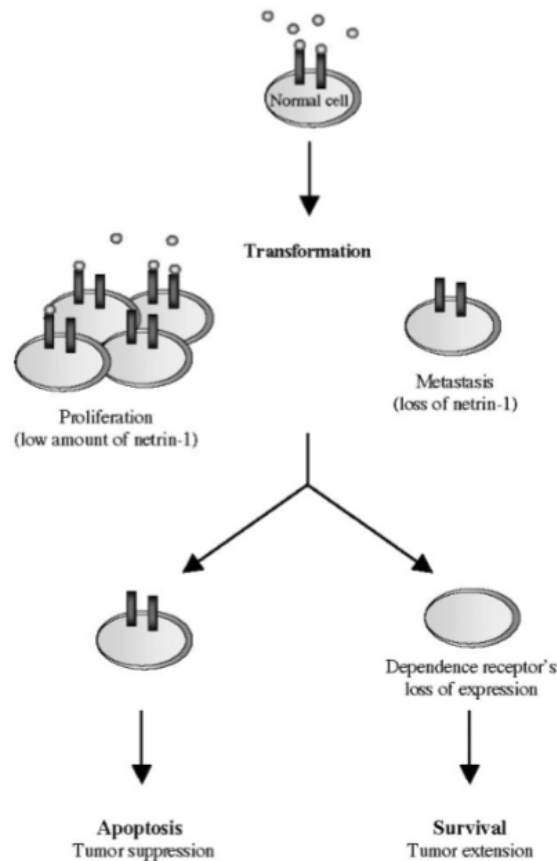
In the presence of Netrin-1, UNC5B and UNC5B are dimerized, and their intracellular domain adopts a close conformation in which ZU5 and death domains interact with each other. DAPK interacts with the closed intracellular domain, but is in an inactive autophosphorylated state. Following ligand withdrawal, UNC5H 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 allow interaction between the DD of UNC5H and DAPK, thus leading to DAPK activation and initiation of an apoptotic program (Goldschneider and Mehlen, 2010).

#### **2.5.4 Role of DCC/UNC5H-induced death in the control of tumorigenesis**

In a normal context a cell expresses DCC and UNC5H in the setting of Netrin-1 presence. On acquiring a transformed phenotype, this cell migrates toward other tissues (metastasis) or proliferates. In both cases, Netrin-1 concentration decreases in regard to DCC or UNC5H expression, either because Netrin-1 is not expressed in other tissues or because the number of transformed cells expressing DCC/UNC5H has increased while netrin-1 concentration remains unchanged. This turns DCC/UNC5H into a death inducer, hence limiting tumor extension. Such a mechanism cannot occur if the cell has acquired the selective advantage of losing/reducing/mutating DCC/ UNC5H expression (Mehlen and Furne, 2005).

Ligand Netrin-1 was found to be overexpressed in a large variety of tumors, like breast cancer, lung cancer, ovarian cancer, neuroblastoma, and medulloblastoma (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Fitamant et al., 2008; Papanastasiou et al., 2011). Tumor cells can turn off the pro-apoptotic signaling by the loss of receptors (Bernet et al., 2007) or by increase Netrin-1 production and autocrine secretion which promotes tumor growth and metastases. Research discovered that in tumor silencing of Netrin-1 can induce cell apoptosis in tumor cells (Delloye-Bourgeois et al., 2009a; Fitamant et al., 2008; Paradisi et al., 2009) (Figure 16).



**Figure 16.** DCC and UNC5H receptors may function as tumor suppressors (Mehlen and Furne, 2005).

## 2.6 An innovative therapeutic target

Considering all the properties of DRs, overexpression of ligands can block DR-mediated cell death induction. There are some cases several DRs share the same ligand. For example, Netrin-1 binds to DCC and UNC5A, B, C, D receptors, while SHH binds to Patched and CDON receptors. So, overexpression of ligand could block cell death induced by several DRs, making this special ligand a controller of cell survival as well as an attractive target in cancer treatment.

According to this theory, in our lab, a humanized anti-Netrin-1 antibody (NP137) was developed to disrupt the interaction between Netrin-1 and Netrin receptors. We identify the V-2 domain of Netrin-1 to be important for its interaction with the Ig1/Ig2 domains of UNC5H2. We generate a humanized anti-netrin-1 antibody that disrupts the interaction between netrin-1 and UNC5H2 and triggers death of Netrin-1-expressing tumor cells *in*



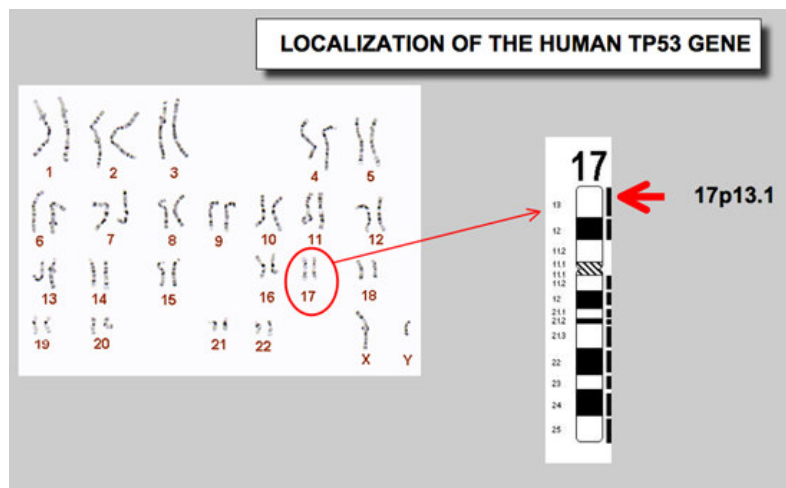
*vitro* (Grandin et al., 2016b). Interestingly and promisingly, after test, no clinical, hematological, or biochemical signs of toxicity were noted in mice and monkeys who received treatment of NP137. Recently, the Phase I clinical trial for NP137 will assess the safety, tolerability, pharmacodynamics, pharmacokinetic and preliminary antitumor activity in patients with locally advanced or metastatic solid tumors. This study will be completed soon and the results are very promising and eagerly awaited.

But in some tumor cell lines and specimens, Netrin-1 is expressed at low level, our lab previous study showing that treatments with conventional drugs are often associated with an upregulation of both Netrin-1 and its receptors, probably because Netrin-1 and its DRs are direct transcriptional targets of p53. Consequently, combining conventional drugs like Doxorubicin with Netrin-1 interference increases tumor cell death *in vitro* and potentiates tumor growth inhibiting effect *in vivo* (Paradisi et al., 2013). This phenomenon suggests that even though when tumors do not express high levels of Netrin-1, a combination of conventional drugs plus drugs inhibiting Netrin-receptors interaction could amplify the response.

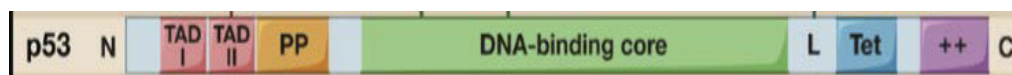
### 3. TP53 family

#### 3.1 TP53 structure

The TP53 gene is located on human chromosome 17 (short arm, 17p13), a region that is frequently deleted in human cancer (Baker et al., 1989) (Figure 17). The human p53 protein domains include the transcriptional activation domain I (TAD I, residues 20–40), the transcriptional activation domain II (TAD II, residues 40–60), the proline domain (PP, residues 60–90), the sequence specific core DNA-binding domain (DNA-binding core, residues 100–300), the linker region (L, residues 301–324), the tetramerization domain (Tet, residues 325–356), and the lysine-rich basic C-terminal domain (+++, residues 363–393) (Vousden and Prives, 2009) (Figure 18).



**Figure 17.** Localization of human p53 gene (Baker et al., 1989).

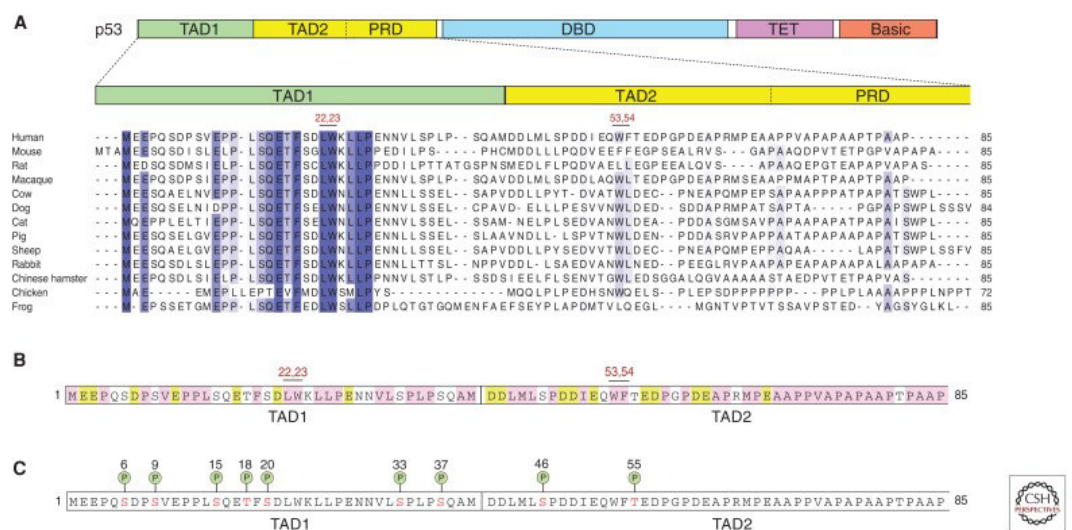


**Figure 18.** Schematic representation of structure of p53 protein domains (Vousden and Prives, 2009).

The N-terminal region of p53 is natively unfolded, although a residual secondary structure is observed in regions of functionally important hydrophobic residues. It consists of an acidic TAD, which further subdivided into two subdomains TAD1 (residues 1–40) and TAD2 (residues 40–61), and a proline-rich region (residues

64–92) (Joerger and Fersht, 2008). Biochemical and structural studies have identified factors that bind either or both TADs, including general transcription factors (GTFs), chromatin modifiers, and negative regulators, helping to elaborate a model through which p53 activates transcription, for example by interacts with the carboxy-terminal portion of the CBP protein, which implicated in cell proliferation and differentiation (Gu et al., 1997). 13–23 amino acids are conserved in different species (Levine, 1997) (Figure 19).

The proline-rich domain (PP) that links the TAD to the DNA-binding domain contains five PXXP motifs, mediating protein-protein interactions in signal transduction through binding to Src homology 3 domains. These motifs are not conserved, but the prevalence of prolines in this region indicating a certain degree of rigidity functionally or structurally (Joerger and Fersht, 2008).

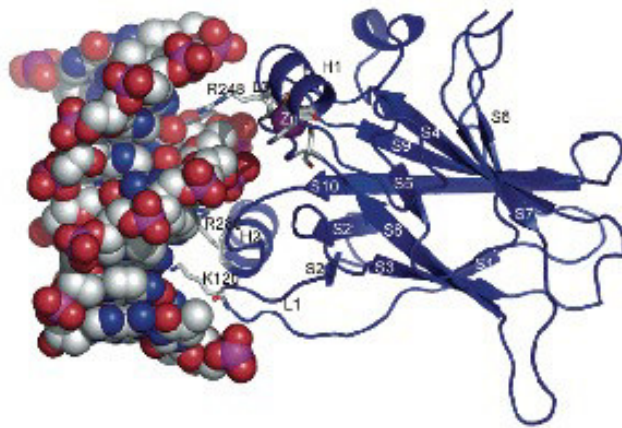


**Figure 19** p53 TAD.

**A:** Protein sequence alignment of the p53 TAD. **B:** Human p53 TAD residues contains acidic (yellow) and hydrophobic amino acids (pink). The four key hydrophobic residues at positions 22, 23, 53, and 54 that are critical for TAD transcriptional activity. **C:** Human p53 TAD residues 1–85 indicating serine and threonine residues that are targets of phosphorylation and affect p53 TAD interactions with binding partners (Raj and Attardi, 2017).

The DNA-binding domain of p53 is localized between amino acid residues 102 and 292. DBD contains a Zn<sup>2+</sup>, which has been shown to be important for aggregation and sequence-specific DNA binding. The tetrameric p53 bind to four repeats of a consensus DNA 5′-PuPuPuC(A/T)-3′ sequence. This sequence is repeated in two

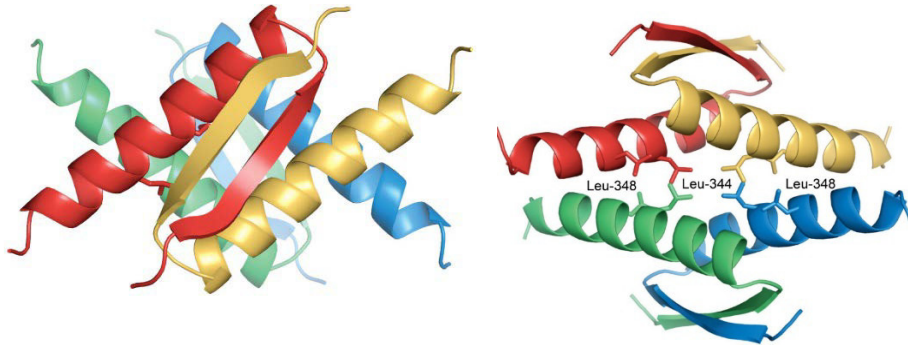
pairs, each arranged as inverted repeats such as  $\rightarrow\leftarrow\rightarrow\leftarrow$ , where  $\rightarrow$  is the sequence given above. The crystal structure of p53 DBD revealed a large-sandwich scaffold, positioning the DNA-binding loop-sheet-helix motif in the major groove and loop L3 in the minor groove (Figure 20). Further, most of the cancer-related mutations are mapped to the DBD (Duan and Nilsson, 2006).



**Figure 20.** Cartoon of the p53 DBD-DNA complex.

The DNA molecule is shown as spheres, DBD is in a cartoon representation,  $Zn^{2+}$  ion-coordinating residues are shown as sticks (Duan and Nilsson, 2006). Residues K120, S241, R273, A276, and R283 make contacts with the phosphate backbone in the major groove, while K120, C277, and R280 interact via hydrogen bonds to the DNA bases. R248 then makes multiple hydrogen bond contacts in the minor groove of the DNA helix (Cho et al., 1994).

Full-length p53 reversibly forms tetramers via a tetramerization domain in the C-terminal region of the protein (residues 323–356) (Veprintsev et al., 2006). The region that forms the tetramerization domain in human p53 is highly conserved among other p53 family members, for example p63 and p73 (Joerger et al., 2014). The tetrameric structure of the domain can be described as a dimer of primary dimers. The monomeric tetramerization domain contains of a short  $\beta$ -strand and an  $\alpha$ -helix linking by a sharp turn, two monomers form a primary dimer, stabilized by an antiparallel intermolecular  $\beta$ -sheet and antiparallel helix packing (Figure 21). The two dimers are held together by a large hydrophobic surface of each helix pair, which then forms a four-helix bundle (Jeffrey et al., 1995).



**Figure 21** The tetrameric structure of the domain, revealed by X-ray crystallography, and in solution by nuclear magnetic resonance. p53 tetramerization domain looks like a dimer of dimers, shown in two different orientations (Jeffrey et al., 1995; Joerger and Fersht, 2008).

The C-terminal 26 amino acids, residues 363–393, form an open (protease-sensitive) domain composed of nine basic amino acid, conferring on p53 the ability to oligomerize and to bind both single-stranded DNA (ssDNA) and RNA (Lee et al., 1995). The C-terminal region of p53 is subject to posttranslational modifications, including acetylation, ubiquitination, phosphorylation, sumoylation, methylation, and neddylation, that regulate p53 function and cellular protein levels (Bode and Dong, 2004; Lavin and Gueven, 2006; Toledo and Wahl, 2006). Acetylation of C-terminal lysine residues in the p53 tumor suppressor is associated with increased stability and transcription factor activity (van Leeuwen et al., 2013). Acetylation of C-terminal lysine residues has been associated with recruitment of Coactivators/Histone Acetyltransferases required for transcriptional activation of target genes (Barlev et al., 2001; Mujtaba et al., 2004).

The amount of p53 protein vary dramatically from one cell type to another, and it will increase rapidly facing certain type of physiologic stress. p53 is a protein with low stability as demonstrated by experiments with cycloheximide. When we treat cells harboring WT p53 with cycloheximide, p53 protein disappeared with a half-life of only 20 minutes, indicating that p53 is normally not stable and fast degraded after it translated.

### 3.2 TP53 discovery and signals provoking p53 induction

As we know, in almost all cells in mammalian tissues, the p53 protein serves as the local representative of the organism's interests to ensure that all the cell keeps its

household in order (Weinberg, 2013). The initial functional studies of p53 involved a substantial scientific detour: firstly, p53 came to be classified as a tumor antigen. The nuclear phosphoprotein p53 was originally discovered in extracts of transformed cells, reacting with antiserum from animals with tumors induced by simian virus 40 (SV40). It was found to form an oligomeric complex in SV40-transformed cells with the SV40 oncogene product, the large T antigen. Because large T antigen is needed to maintain the transformed phenotype, it was suggested that this interaction is important for transformation. Thus, p53 came to be classified as a tumor antigen (Lane, 1979; Levine, 1979). p53 was also detected in tumor-derived or transformed cell lines in culture (DELEO\*, 1979).

For this reason, p53 has been classified as an oncogene. Indeed, transfection of a p53 cDNA clone into rat embryo fibroblasts revealed that this DNA could collaborate with a co-introduced ras oncogene in the transformation of these rodent cells. Co-transfection indicates that the p53-encoding gene can play a causal role in the conversion of normal fibroblasts into tumorigenic cells. In addition, a variety of genomic and complementary DNA clones of p53 were isolated that could immortalize cells in culture (Jenkins et al., 1984). A variety of observations suggested that the p53 gene might operate as an oncogene (Parada et al., 1984; Weinberg, 2013).

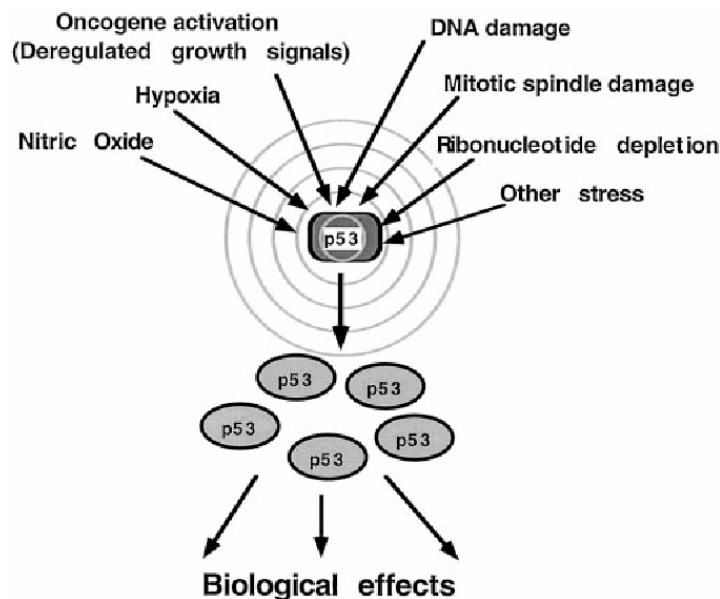
However, later p53 was classified as a tumor suppressor. Indeed, all of above transforming p53 cDNA clones turned out to be the mutant forms of p53 (Hinds et al., 1989). The p53 cDNA had originally been synthesized using as template the mRNA extracted from tumor cells, rather than normal cells, then an cDNA cloned from this mRNA, actually suppressed cell transformation, rather than helping it. The only difference between these two cDNAs is a single based substitution: a point mutation, which induced an amino acid change in p53 protein, eventually encoding a mutant p53 protein whose function totally altered (Eliyahu et al., 1989; Finlay et al., 1989; Michalovitz et al., 1990). In contrast, WT p53 acts as a tumor suppressor, negatively regulating cell cycle and suppressing cell transformation (Finlay et al., 1989) and the growth of tumor cells (Baker et al., 1990).

However, later studies showed that p53 is not a typical tumor suppressor gene. Indeed, most of the other tumor suppressor genes are inactivated by frameshift or nonsense mutations leading to disappearance or aberrant synthesis of the gene product, almost 90% of p53 gene mutations are missense mutations leading to the synthesis of a stable protein, lacking its specific DNA binding function and accumulating in the nucleus of tumor cells (Soussi and Beroud, 2001). For typical tumor suppressor gene, deletion in mouse germ line lead to defects of embryonic development, indicating that these typical tumor suppressor gene function as negative regulators of proliferation. On the contrary, deleting p53 from mouse germ line has no obvious effect on cell proliferation during development. Indeed, mice lacking p53 gene have a short life span and develop lymphomas and sarcomas, clearly indicating that p53 is a tumor suppressor gene. These observations suggest that p53 prevents the appearance of abnormal cells, especially those cells that were capable of spawning tumors (Lozano, 2010).

In the early 1990s, it was found that X-rays, ultraviolet (UV) radiation, some chemotherapeutic drugs that damage DNA, inhibitors of DNA synthesis, and agents that disrupt the microtubule components of the cytoskeleton are able to induce DNA damage resulting accumulation as well as activation of p53 in short time (Maltzman and Czyzyk, 1984; Rubtsova et al., 1998; Zhan et al., 1993). Increase of p53 protein levels can be induced by several cell physiologic signals. Low oxygen tension (hypoxia) is experienced by cells, normal and malignant, that lack adequate access to the circulation and thus to oxygen borne by the blood (Van Meir, 1996). Still later, introduction of either the adenovirus E1A or *myc* proto-oncogene into cells was also found to be capable of causing increases in p53 levels. Moreover, p53 may act as a protector of cell against the formation of cancers. Many tumors begin to replicate and reach a critical size when the blood supply becomes rate-limiting, requiring angiogenic factors to sustain growth. Hypoxia was revealed triggering p53 activity and killing these cells, and eliminating tumor growth (Humpton and Vousden, 2016).

Now, we have discovered different stimulus that can induce p53, for examples, oxidative stress (Peuget et al., 2014), ionizing radiation (Khanna and Lavin, 1993), ribonucleotides starvation (Linke et al., 1996), heat shock (Han et al., 2013), nitric

oxide exposure (Forrester et al., 1996) and activated oncogenes (Okaichi et al., 2012) (Figure 22). This rapid induction occurred in the absence of any marked changes in p53 mRNA levels and hence was not due to increased transcription of the p53 gene. Instead, it soon became apparent that the elevated protein levels were due entirely to the post-translational stabilization of the normally labile p53 protein. In normal condition, cell can continuously synthesize p53 at a high rate and fast degrade them at an equal speed. However, in response to some physiologic signals or stress, the degradation of p53 is blocked, resulting in a rapid increase of p53 levels in the cell. Numerous demonstrates a diverse array of sensors are responsible for monitoring the integrity and functioning of various cellular systems. When these sensors detect damage or aberrant functioning, they send signals to p53 and its regulators, resulting in a rapid increase in p53 levels within a cell (Eischen, 2016; Lavin and Gueven, 2006; Oren, 1999; Yang et al., 2004). How p53 can be stabilized and activated will be discussed in the next following chapter.



**Figure 22.** Signals that activate p53. Activation results in markedly increased overall p53 protein levels and most probably also in qualitative changes in the protein (Oren, 1999).

### 3.3 Stabilization of p53 levels and post-translational modification

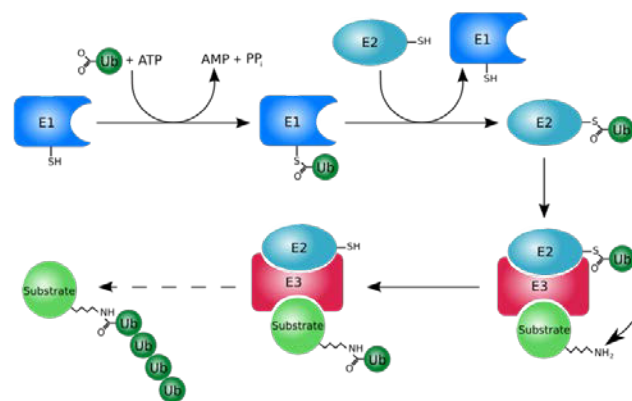
Over these years, numerous post-translational modifications of p53 have been identified, and the roles of different modification have been extensively studied.



Ubiquitination, sumoylation and neddylation have been reported to suppress p53-mediated transcription and nuclear export. Phosphorylation and acetylation of p53 have been shown to mainly promote the expression of p53 transcriptional targets, Moreover, methylation, has been reported associated with both enhancement and repression of p53 function, dependent on the specific site of methylation (Lee and Gu, 2010).

### a. Ubiquitination

Ubiquitin is a small regulatory protein, 76-amino acid polypeptide, found in most tissue of eukaryotic organisms. It was found in 1975 (Goldstein et al., 1975). Ubiquitination is a reversible posttranslational modification of cellular proteins, in which ubiquitin, is primarily attached to the epsilon-amino group of lysine residues in target proteins. Finally mark the protein for degradation via proteasome, altering the location in cell, affect the activity and promote or prevent protein interaction (Mukhopadhyay and Riezman, 2007). Proteins ubiquitylated in a series of enzymatic reactions catalyzed by enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase) (Glickman and Ciechanover, 2002). The ubiquitin-ligases comprise a large and diverse group of proteins, sometimes functioning in large multiprotein complexes, and these are the enzymes that are responsible for determining the substrate specificity of the ubiquitin pathway (Figure 23).

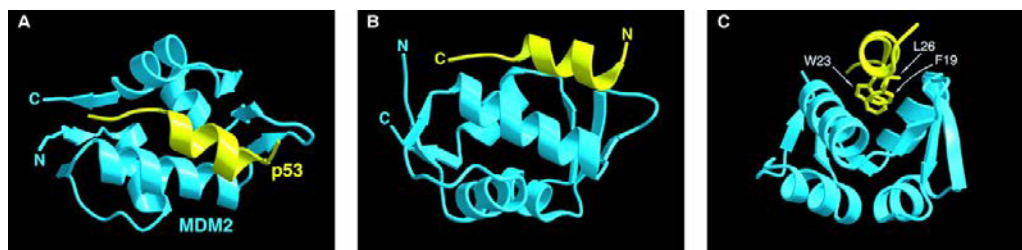


**Figure 23** Schematic representation of ubiquitination system.

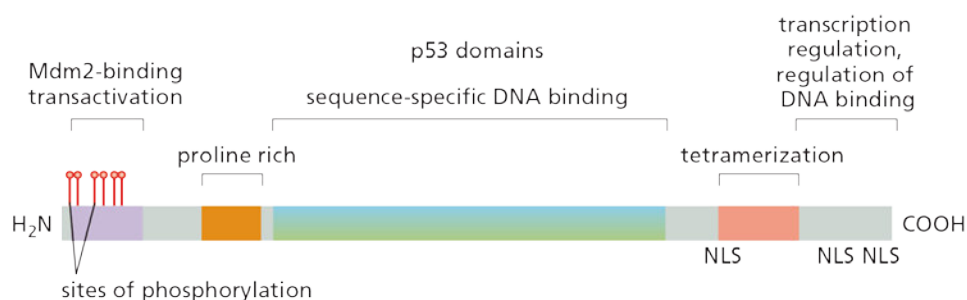
E1, ubiquitin-activating enzyme, activates ubiquitin by forming a thiol ester link between the carboxy terminus Gly-76 of ubiquitin and the Cys of E1 in an ATP-dependent manner. The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme, also through a thiol ester bond. E2 interacts with E3 ubiquitin ligase, that recognizes the substrate. Finally, ubiquitin can be transferred to its target, forming a covalent isopeptide bond between the carboxyl terminus Gly76 of ubiquitin and a primary amine of the target protein (McDowell and Philpott, 2013).

p53 is normally maintained at low levels in unstressed mammalian cells by continuous ubiquitination and subsequent degradation by the 26S proteasome. This is primarily due to the interaction of p53 with MDM2 (Mouse double minute 2 homolog, also known as E3 ubiquitin-protein ligase MDM2).

MDM2 (MDM2 proto-oncogene) protein is a key player in the regulation of p53; it is the product of an oncogene, often hyper-activated in several types of human cancer (Zhao et al., 2014). MDM2 was first cloned as a gene amplified on double minute particles in a transformed murine cell line, hence its name, murine double minute 2 (Lozano and Montes de Oca Luna, 1998). MDM2 rose to fame after identification as a p53 binding protein, and highly expression in many cancers. There are two functions involving the binding between p53 and MDM2: 1) interaction with and inhibition of p53, precise mechanisms will be described after; 2) shuttle p53 from the nucleus to the cell cytoplasm, where p53 can be recognized and degraded by proteasome. Interference binding of the p53/MDM2 complex in normal cells results in activation of p53 signaling. Crystal structure of the 109 amino acid N-terminal region of MDM2 bound to a 15 amino acid peptide derived from the transactivation domain of p53. The p53 peptide formed an amphipathic-helix configuration, hiding the key hydrophobic side chains of phenylalanine-19, tryptophan-23, and leucine-26 deep in a hydrophobic pocket on the surface of MDM2 (Ito et al., 2001; Kane et al., 2000; Kussie et al., 1996). (Figure 24)



D



**Figure 24.** The MDM2 N-terminal domain (in cyan) forms a structure reminiscent of a twisted trough. It has a hydrophobic cleft where the p53 peptide (in yellow) binds as an amphipathic  $\alpha$  helix. Three approximately orthogonal views of the complex are shown.

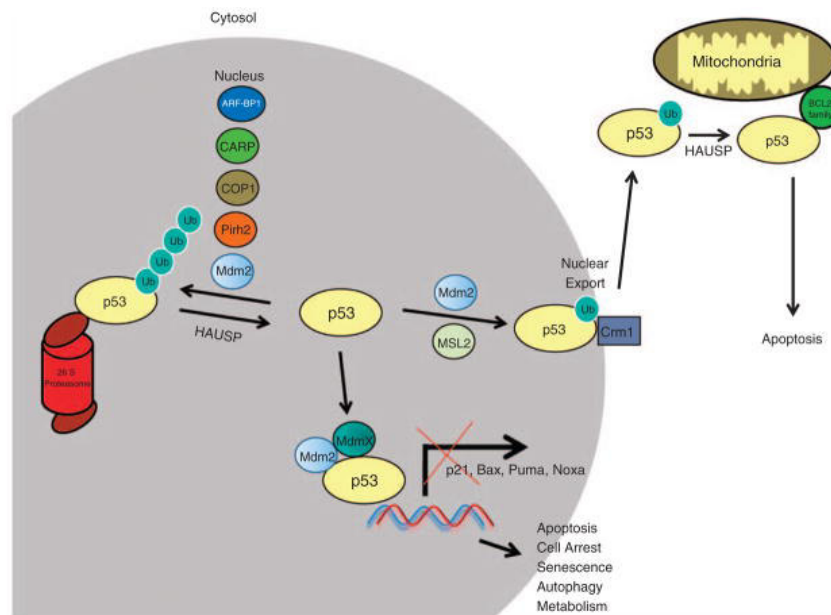
**A)** The MDM2-p53 complex in an orientation where the floor of the MDM2 cleft is in the plane of the figure. **B)** The complex rotated approximately  $90^\circ$  about the horizontal axis of figure A. **C)** The complex rotated approximately  $90^\circ$  about the vertical axis of (B), looking down the helix axis of p53 (Kussie et al., 1996). **D)** The interaction of p53 with MDM2 occurs in a small domain near its N-terminus, where the transactivation domain of p53 is also located. The phosphorylation of p53 amino acid residues in this region (red lollipops) blocks Mdm2 binding and thus saves p53 from ubiquitylation and degradation (D.E. Fisher, 2000).

MDM2 have roles in both localization and degradation of p53 (Alarcon-Vargas and Ronai, 2002). Data indicates that low levels of MDM2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote p53 polyubiquitination and nuclear degradation, thus determining the fate of p53 (Li Mingyuan, 2003). MDM2 directs ubiquitination of p53 to the lysine residues in the C-terminus, allowing the nuclear export signal (NES) to be exposed. In this way, p53 protein can be exported from nucleus to cytoplasm, subsequently polyubiquitylated and degraded in cytoplasmic by the proteasome pathway rapidly (Lee and Gu, 2010) (Figure 25). However, when cells are facing the genotoxic stress, to achieve p53 accumulation it should be protected from MDM2, and this kind of protection can be achieved by phosphorylation of p53, which can block MDM2 binding. p53 phosphorylation is achieved by activation of kinases such as ATM (ataxia telangiectasia mutated) and Chk1 (Checkpoint Kinase1) (Ou et al., 2005; Saito et al., 2002). ATM, a protein that response to DNA damage, regulates MDM2 protein. After rapidly activated by DNA damage, ATM can phosphorylate at Ser15, and these modifications inhibit the ability of MDM2 to polyubiquitinate p53, thus finally leading to increased p53 (Cheng and Chen, 2010; Saito et al., 2002). Chk1, which phosphorylates p53 at Ser20 in N-terminal domain, change p53 N-terminal domain, where interaction with MDM2 happens, preventing the contacting of MDM2 and p53 (Kulkarni and Das, 2008). Following phosphorylation of both p53 and MDM2 proteins, MDM2 fails to initiate ubiquitylation of p53, which can escape from degradation, and rapidly accumulate in the cell (Bode and Dong, 2004).

In the contrary, cancer cells also express proteins that can be in direct opposition by de-ubiquitylation of p53. For example, protein HAUSP (Herpes-virus-

associated ubiquitin-specific protease) is able to bind p53 (Wood, 2002). It has been shown that overexpression of HAUSP reduces the amount of ubiquitylated p53, and moreover, HAUSP is also involved in regulation of p53 independently by MDM2 (Tavana and Gu, 2017). Another mechanism that affects MDM2 has been revealed through the discovery of an MDM2 antagonist: ARF (ADP-ribosylation factor). ARF is able to bind with MDM2 and inhibits its action, either by sequestering MDM2 in the nucleolus (the nuclear structure that is largely devoted to manufacturing ribosomal subunits) or by inhibiting MDM2 in the nucleoplasm (Midgley et al., 2000). Moreover, MDM2 itself is transcriptionally-regulated by p53. Thus, MDM2 can regulate its own levels through auto-ubiquitination. This duality defines a negative feedback loop, which probably serves to keep p53 in tight check and to terminate the p53 signal once the triggering stress has been effectively dealt with (Alarcon-Vargas and Ronai, 2002).

Although MDM2 is a key regulator of p53 stability, other mechanisms for p53 ubiquitination and degradation also exist. For example, it has been shown a role of the c-Jun N-terminal kinase (JNK). The binding of JNK to p53 results in ubiquitination and proteolytic removal of p53 (Fuchs et al., 1998). Under unstressed conditions, JNK binds to p53 preferentially in the G0/G1 cell-cycle phase and the interaction results in the ubiquitylation and degradation of p53. Conversely, under stress conditions, JNK phosphorylates p53 at threonine 81, resulting in stabilization and activation of p53 (Fuchs et al., 1998).



**Figure 25.** Ubiquitinated p53 functions and pathways.

Low levels of MDM2 have been shown to induce mono-ubiquitination and target p53 for nuclear export. p53 C-terminal ubiquitination mediated by MDM2 was identified to be essential for the nuclear export. A p53-ubiquitin fusion protein, with an ubiquitin fused to C-terminus, was shown to localize in the cytosol suggesting ubiquitin could expose the NES that is normally masked when p53 is in a tetramer, then NES is recognized by a nuclear export regulator, and exported to cytosol (Lee and Gu, 2010).

## b. Sumoylation

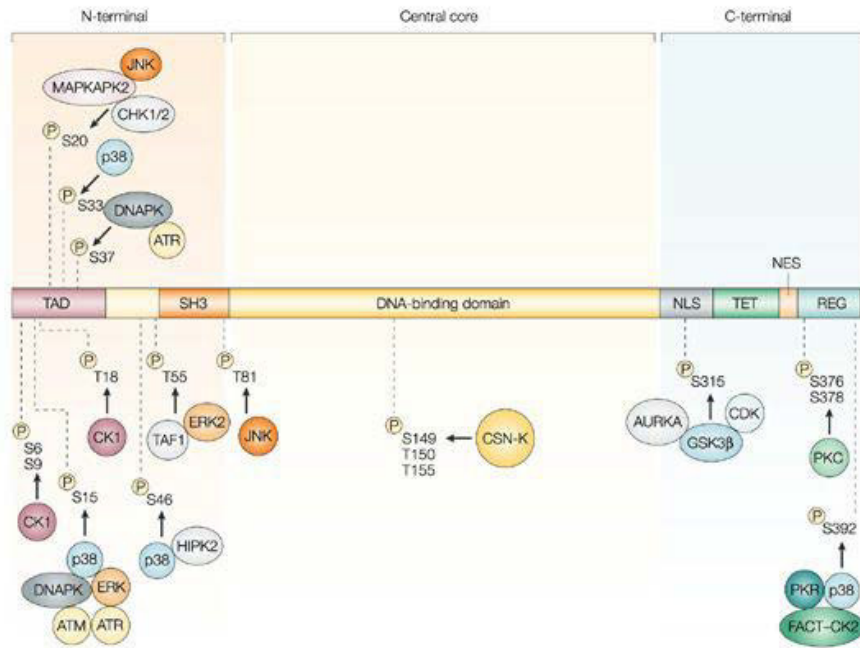
Sumoylation is similar to ubiquitylation, as an isopeptide bond is formed between the C-terminal carboxy group of the small ubiquitin-like protein SUMO1 and the  $\epsilon$ -amino group of a lysine residue in the target protein. The target for sumoylation in p53 is Lys386 (Rodriguez et al., 1999). Sumoylation modulate p53 transcriptional activity. Monica Gostissa and colleagues have shown that conjugation of SUMO-1 to wild-type p53 results in an increased transactivation ability of p53 (Gostissa et al., 1999), however, some data show that sumoylation of p53 has a repressive effect, and p53 sumoylation is regulated by MDM2- and ARF-mediated nucleolar targeting (Chen and Chen, 2003). Until now whether sumoylation results in activation or repression of p53 activities is still controversial.

### **c. Acetylation**

Acetylation is a reaction that introduces an acetyl functional group into a chemical compound, on the contrary, deacetylation is the removal of this acetyl group. Acetylation of p53 is an important reversible enzymatic process that occurs in response to DNA damage and genotoxic stress and is indispensable for p53 transcriptional activity. C-terminal lysine residues at positions 164, 305, 370, 372, 373, 381, and 382 have been shown to be acetylated by the HAT p300/CREB-binding protein (CBP) and lysine 320 by the HAT p300/CBP-associated factor (PCAF) (Marouco et al., 2013). Acetylation of these sites is important as a docking site for the subsequent recruitment of HAT and other transcription co-factors to promoter regions. Indeed, p300/CBP can enhance histone acetylation and promote transcription in the vicinity of target genes (Brooks and Gu, 2011). p53 DNA binding domain is acetylated in lysine at position 120, and hMOF and TIP60 are responsible for this acetylation. The consequence of this acetylation is that it affects accumulation and activation of pro-apoptotic genes, such as PUMA and BAX (Sykes et al., 2006). In addition, deacetylation of Lys-382 by SIRT1 (also known as sirtuin, silent mating type information regulation 2 homolog), an enzyme that deacetylates proteins that contribute to cellular regulation, impairs its ability to induce pro-apoptotic program and modulate cell senescence. Deacetylation by SIRT2 impairs its ability to induce transcription activation in an AKT-dependent manner (Bordone and Guarente, 2005; Luo et al., 2001; Vaziri et al., 2001).

### **d. Phosphorylation and de-phosphorylation**

Phosphorylation is the chemical addition of a phosphoryl group ( $\text{PO}^{3-}$ ) to an organic molecule. The removal of a phosphoryl group is called de-phosphorylation. 17 phosphorylation/de-phosphorylation sites have been found (Figure 26).



**Figure 26** Phosphorylation sites of human p53.

The most common post-translational modification of p53 is phosphorylation. 17 serine and threonine sites have been reported to be phosphorylated on p53. Most of the sites are located in the amino-terminal region. Some protein kinases phosphorylate numerous sites and several sites are phosphorylated by more than one protein kinase. **ATM**, ataxia telangiectasia mutated; **ATR**, ataxia telangiectasia and Rad3 related protein; **AURKA**, aurora kinase A; **CDK**, cyclin-dependent kinase; **CHK**, checkpoint kinase; **CK**, casein kinase; **CSN-K**, COP9 signalosome associated kinase complex; **DNAPK**, DNA-dependent protein kinase; **ERK**, extracellular signal-regulated kinase; **GSK3 $\beta$** , glycogen synthase kinase-3 $\beta$ ; **HIPK2**, homeodomain-interacting protein kinase 2; **JNK**, c-JUN N-terminal kinase; **MAPKAPK2**, mitogen-activated protein kinase-activated protein kinase 2; **NES**, nuclear export signal; **NLS**, nuclear localization signal; **p38**, p38 kinase; **PKC**, protein kinase C; **REG**, carboxy-terminal regulatory domain; **SH3**, Src homology 3-like domain (Bode and Dong, 2004).

In humans, serine residues 6, 9, 15, 20, 33, 37 and 46 and threonine residues 18 and Thr81 in the amino-terminal region, serine residues 315 and 392 in the C-terminal domain, threonine residues 150 and 155 and serine residue 149 in the central core are all p53 phosphorylation sites (Ashcroft et al., 1999; Bode and Dong, 2004). Studies also highlighted two highly conserved phosphorylation sites in p53: the serine residues 20 and 392 site, forming a paradigm of how phosphorylation controls p53 transcriptional function (Maclaine and Hupp, 2009).

The Ser392 phospho-acceptor site is located in the C-terminal regulatory domain in a flexible and unstructured motif, whose phosphorylation by casein kinase 2 (CK2) stimulates the sequence-specific DNA-binding function of p53 (Hupp et al., 1995). Phosphorylation p53 at Ser392 also increases after either UV or ionizing radiation in cell lines, in mice splenocytes *in vivo*, and in human skin basal cell

populations (Blaydes and Hupp, 1998; Finlan et al., 2006). These data are consistent with an activating rather than inhibitory role for phosphorylation of this site on p53 function. The second highly conserved phospho-acceptor site, Ser20, is located in the N-terminal TAD in an unstructured linear motif whose phosphorylation stabilizes the binding of the transcriptional co-activator CREB-binding protein (p300) by creating a phospho-SDLxxLL docking motif (Dornan et al., 2003). The docking of p300 to this motif is required to promote DNA-dependent acetylation of p53 at promoters, and hence transcriptional activation of p53 target genes (Dornan et al., 2003). Mutation of Ser20 to Asp20, thereby mimicking constitutive phosphorylation of p53 Ser20, results in a p53 with enhanced transcription function in cell lines, suggesting that phosphorylation of p53 at Ser20 forms a stimulatory rather than an inhibitory signal for p53 activity (Jabbur et al., 2001; Jabbur and Zhang, 2002).

### **e. Methylation**

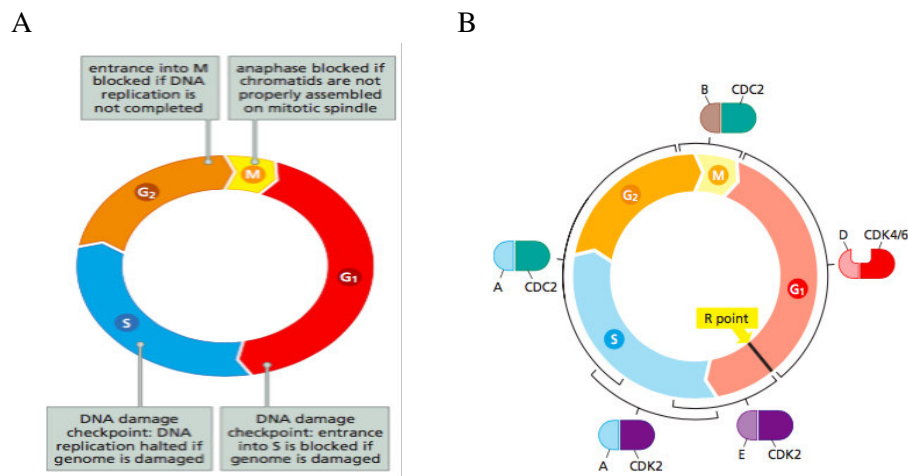
Several studies have established that protein methylation is a novel mechanism by which p53 is regulated. Lysine and arginine residues can be modified by methylation. For example, histone lysine methyltransferases KMT5 (Set9), KMT3C (Smyd2), and KMT5A (Set8) methylate p53 at specific C-terminal lysine residues. Lysine methylation enhances or suppresses p53 transcriptional activity depending on the methylation site (Scoumanne and Chen, 2008). Also, protein arginine methyltransferase (PRMT) 5, a co-factor in a DNA damage responsive co-activator complex that interacts with p53, is responsible for p53 methylation. Arginine methylation is regulated during the p53 response and affects the target gene specificity of p53. PRMT5 depletion triggers p53-dependent apoptosis. Thus, methylation on arginine residues is an underlying mechanism of control during the p53 response (Jansson et al., 2008).



### 3.4 p53 functions

#### 3.4.1 p53 functions as a transcription factor, blocking cell cycle and attempts to repair DNA damage

The downstream events mediated by p53 take place by two major pathways: cell cycle arrest and apoptosis. The mammalian growth-and-division cycle is divided into four phases— G1 (growth); S phase (DNA synthesis); G2 (growth); and mitosis (cell and nuclear division). A fifth stage resides outside the proliferative cycle and is known as quiescence (G0), where cells can reversibly exit the cell cycle (Barr and Mansfeld, 2019). Cell deploys a series of surveillance mechanisms that monitor each step during cell cycle progression and permit the cell to proceed to the next step in the cycle only if a prerequisite step has been completed successfully. If specific steps in the execution of a process go awry, these monitors rapidly call a halt to further advance through the cell cycle until these problems have been successfully addressed, and these monitoring mechanisms are called checkpoints. Specific steps of the cell cycle are controlled by changing the levels and availability of cyclins. Cyclins function by activating the catalytic function of their partners, the cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases. Additional control of the cell cycle is provided by CDK inhibitors, which antagonize the activities of cyclin-CDK complexes (Weinberg, 2013) (Figure 27).



**Figure 27** examples of checkpoints in the cell cycle and pairing of cyclins with cyclin-dependent kinases.

**A)** cell will not be permitted to enter into S phase until all the steps of G1 have been completed. It will be blocked from entering G2 until all of its chromosomal DNA has been properly replicated. Cell is not permitted to enter into anaphase until all of its chromosomes are properly assembled on the mitotic spindle during metaphase. Cell is not allowed to advance into S or M if its DNA has been damaged and not yet repaired. **B)** Each type of cyclin pairs with a specific cyclin-dependent kinase. The D-type cyclins (D1, D2, and D3) bind CDK4 or CDK6, the E-type cyclins (E1 and E2) bind CDK2, the A-type cyclins (A1 and A2) bind CDK2 or CDC2, and the B-type (B1 and B2) bind CDC2. The brackets indicate the periods during the cell cycle when these various cyclin–CDK complexes are active (Weinberg, 2013).

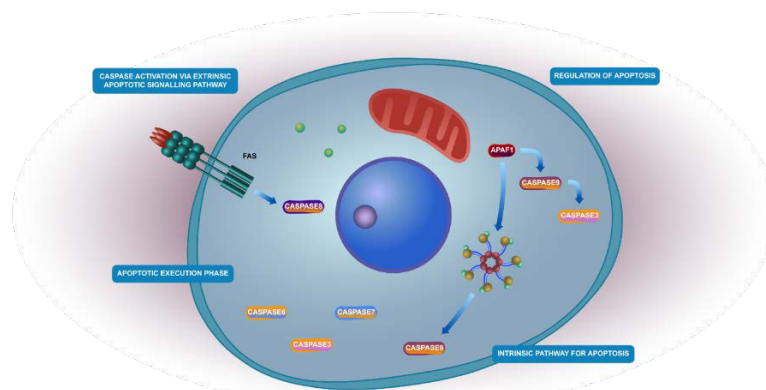
p53 is involved in 3 checkpoints in the cell cycle:

- 1) **G1-to-S phase transition:** p53 can effectively block cell cycle progression by activating the transcription of the cyclin-dependent kinase inhibitor p21, although several other p53-target genes such as 14-3-3 sigma and GADD45 also contribute to this response. As CDK kinase activity is required for various cell cycle transitions, p21 act as a cell cycle inhibitor. Once p21 is activated, it binds to cyclin-CDK complexes and inhibits their kinase activity (el-Deiry, 1998). Moreover, p21 can also binds to the proliferating cell nuclear antigen (PCNA), forming a complex able to block the role of PCNA as a DNA polymerase processivity factor in DNA replication, but not its role in DNA repair inhibiting DNA replication (Levine, 1997). p21 can act on cyclin–CDK complexes and PCNA to stop DNA replication.
- 2) **G2-M checkpoint:** p53-dependent cell cycle arrest at the G2 checkpoint involves inhibition of CDC2, the cyclin-dependent kinase required to enter mitosis. CDC2 is inhibited simultaneously by three different transcriptional targets of p53, GADD45, p21, and 14-3-3s. Binding of CDC2 to Cyclin B1 is required for its activity, and repression of the cyclin B1 gene by p53 also contributes to blocking entry into mitosis (Taylor and Stark, 2001). Interestingly, repression of the topoisomerase II gene by p53 helps to block entry into mitosis and strengthens the G2 arrest (Clifford et al., 2003).
- 3) **G0–G1–S phase transition:** Gas1 (growth arrest specific 1), a membrane-associated protein, is regulated by p53 at the transcriptional level, and it is down regulated during the G0-to-S phase transition in serum-stimulated cells. Gas1 is not expressed in growing or transformed cells, and when overexpressed in normal fibroblasts, it blocks the G0-to-S phase transition. Overexpression of

Gas1 arrests cell proliferation in a p53-dependent manner. In this case, transcriptional activity of p53 is not required. Indeed, inactivated mutations in p53 transcriptional domain did not affect regulation of Gas1 signals for G0 arrest (Del Sal et al., 1995).

### 3.4.2 p53 function as cellular guardian and executioner triggering apoptosis

As described before, the two principal pathways of apoptosis are 1) the Bcl-2 intrinsic pathway induced by various forms of stress, like DNA damages, developmental cues, and external stimuli. 2) the caspase 8/10 dependent or extrinsic pathway initiated by the engagement of death receptors. The caspase 8/10 dependent or extrinsic pathway is a death receptor-mediated mechanism that results in the activation of caspase-8 and caspase-10 (Figure 28).



**Figure 28.** Apoptosis is a distinct form of cell-death, and the mechanisms of various apoptotic pathways (intrinsic pathway and extrinsic pathway) are still being studied till today.

The apoptotic mechanisms of p53 have been intensively dissected and multiple pathways have been identified. Indeed, p53 is able to up-regulate several genes involved in both apoptotic pathways, such as BAX or PUMA (Chipuk and Green, 2006) (Vousden and Prives, 2009). Apoptosis activity is crucial for p53 tumor suppression (Pistritto et al., 2016). A strong link between the apoptotic function of p53 and tumor suppression has been demonstrated using transgenic mice bearing an SV40 large T antigen mutant, which inhibited pRb function without directly compromising p53 activity. These mice develop choroid plexus tumors, but at a

slow rate owing to continuous p53-dependent apoptosis (Saenz Robles and Pipas, 2009).

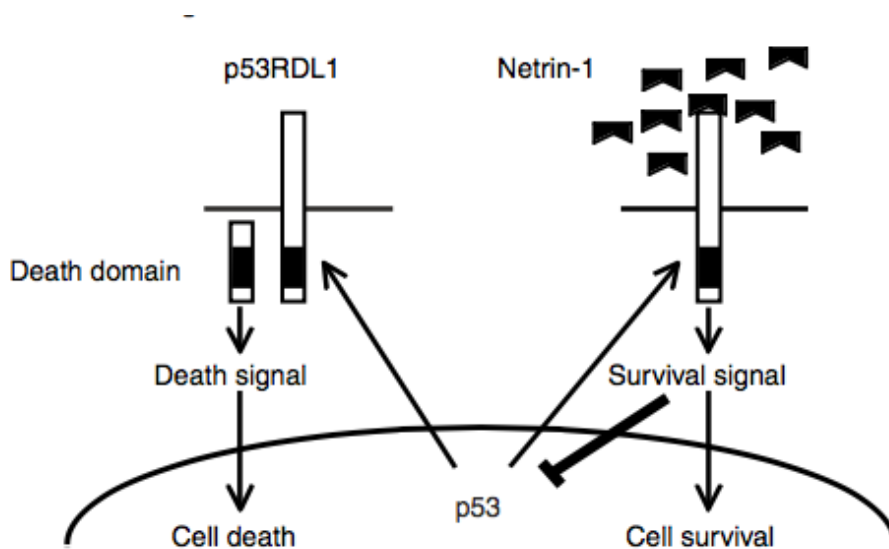
The generation of p53-null mice led to a myriad of experiments to assess p53 functions *in vivo* by comparison to wild-type mice (Donehower et al., 1992; Jacks et al., 1994; Saenz Robles and Pipas, 2009). That p53-null mice succumb to a tumor phenotype, establishing these mice as a viable model to study tumor suppression. As a possible mechanism of tumor suppression, the ability of p53 to initiate apoptosis was examined. In response to irradiation, or drugs such as etoposide, normal thymocytes show a p53-dependent apoptosis response (Clarke et al., 1993; Lowe et al., 1993). The central nervous system of the developing embryo at E12.5 also shows a radiation- and p53-dependent cell-death phenotype (Lee et al., 2001), as does the intestine (Clarke et al., 1993). These experiments established the role of p53 in initiating apoptosis in response to DNA damage, and predicted that loss of apoptosis would contribute to tumor development (Haupt et al., 2003).

### **3.4.3 A new receptor p53RDL1(UNC5B) regulates p53-dependent apoptosis**

During recent years, some papers have described a newly target of p53 that may define a new pathway for p53-dependent apoptosis. The p53RDL1 (p53-regulated receptor for death and life; also termed UNC5B) gene product, corresponding to the Netrin-1 dependence receptor UNC5B, contains a cytoplasmic carboxy-terminal death domain. Interestingly, Chizu Tanikawa and colleagues discovered that p53RDL1 mediates p53-dependent apoptosis (Tanikawa et al., 2003). Similarly, it has been also showed that another Netrin-1 dependence receptor, UNC5D, is a direct transcriptional target of p53. Indeed, during doxorubicin-mediated apoptosis, UNC5D was significantly induced in p53-proficient U2OS cells but not in p53-deficient H1299 cells, suggesting that UNC5D gene is also dependent on p53 status (Wang et al., 2008). Moreover, more recently it has been shown that, in various human cancer cell lines, chemotherapeutic agents such as Doxorubicin, 5-Fluorouracil, Paclitaxel and Cisplatin treatments trigger an increase

of Netrin-1 expression which is accompanied by Netrin-1 receptors increase, and this Netrin-1 upregulation appears to be p53-dependent (Paradisi et al., 2013).

Taken together, above results showing p53 might regulate the survival of damaged cells by balancing the regulation of Netrin–UNC5B signaling, and cell death through cleavage of UNC5B during apoptosis induction (Figure 29).



**Figure 29** A hypothetical model for regulation of cell death and survival through the UNC5B–Netrin-1 interaction in the putative p53-dependent apoptotic pathway (Tanikawa et al., 2003).

### 3.4.4 p53 functions in cell senescence

Cellular senescence is a stress response that links the degenerative and hyperplastic pathologies of aging (Regulski, 2017). Senescent cells exhibit enlarged cell size, flattened morphology, inability to synthesize DNA, metabolic active, and expression of the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal). Further studies also showed that this life timing is controlled by repeats of telomere, a nucleoprotein structure at chromosome tips, which is undergoing progressive loss during cell divisions. Insufficient telomere after a number of cell doublings exposed or fused chromosome ends trigger DNA damage. p53, working as a major mediator of the DNA, has been shown to be critical for telomeric stress-induced cellular senescence. Some stress signals, like aberrant oncogene activation and cancer chemotherapeutic drugs are able to induce senescence-like phenotypes in

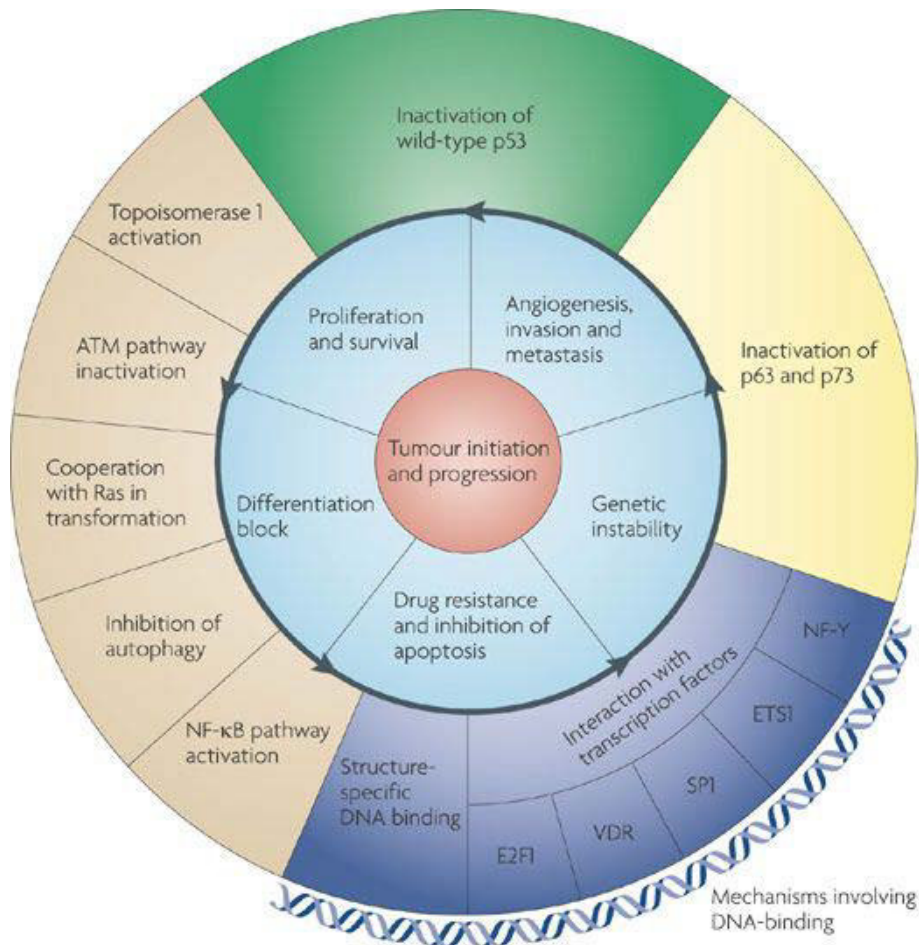
both primary and tumor cells via activating the p53 and/or p16 pathways (Rufini et al., 2013).

## **3.5 p53 and cancer**

### **3.5.1 p53 mutant and tumor formation**

Inactivation of the p53 tumor suppressor is a frequent event in tumorigenesis. As reported, p53 mutations are found in 50–55% of all human cancers, demonstrating the crucial role of p53 in tumor suppression (Hamzehloie et al., 2012) (for further information, see the IARC TP53 mutation database (<http://p53.iarc.fr/TP53SomaticMutations.aspx>). Unlike the majority of tumor suppressor genes, such as RB, APC, or BRCA1, which are usually inactivated during cancer progression by deletions or truncating mutations, the TP53 gene in human tumors is often found to undergo missense mutations, in which a single nucleotide is substituted by another. Consequently, a full-length protein containing only a single amino acid substitution is produced (Hainaut and Hollstein, 2000). The cancer-associated TP53 mutations are very diverse in their locations within the p53 coding sequence and their effects on the thermodynamic stability of the p53 protein. However, the vast majority of the mutations result in loss of p53 ability to bind DNA in a sequence-specific manner and activate transcription of canonical p53 target genes (Bullock and Fersht, 2001). TP53 mutations are distributed in all coding exons, with a strong predominance in exons 4-9, which encode the DNA-binding domain of the protein. Of the mutations in this domain, about 30% fall within 6 “hotspot” residues (residues R175, G245, R248, R249, R273, and R282) and are frequent in almost all types of cancer (Cho et al., 1994). The existence of these hotspot residues could be explained both by the susceptibility of particular codons to carcinogen-induced alterations and by positive selection of mutations that affect cell growth, thus providing survival advantages. In addition to the loss of function that a mutation in TP53 may cause, many p53 mutants are able to actively promote tumor development by several other means. In a heterozygous situation, where both WT and mutant alleles exist, mutant p53 can antagonize WT p53 tumor suppressor functions in a dominant negative (DN) manner. The inactivation of the WT p53 by the mutant p53 in a DN mechanism stems from the

fact that the transcriptional activity of WT p53 relies on the formation of tetramers, whose DNA binding function may be interfered by mutant p53 (Milner et al., 1991; Sigal and Rotter, 2000). However, such a heterozygous state is often transient, as TP53 mutations are frequently followed by loss of heterozygosity (LOH) during cancer progression. LOH is often seen in the case of tumor suppressors where, at a particular locus heterozygous for a mutant and WT allele, the WT allele is either deleted or mutated. The LOH of the short arm of chromosome 17, where TP53 is located (Miller et al., 1986), implies a selective force driving the inactivation of the remaining WT allele, suggesting that the DN activity of mutant p53 is not sufficient to completely inactivate WT p53. Furthermore, data supports the concept that many mutant p53 isoforms can exert additional oncogenic activity by a gain-of-function (GOF) mechanism. GOF refers to the acquisition of oncogenic properties by the mutant protein, compared with the mere inactivation of the protein (Brosh and Rotter, 2009; Oren and Rotter, 2010). Both the DN and GOF effects may play a significant role in the positive selection of missense mutations in TP53 during tumorigenesis (Miller et al., 2016). Mutant p53 oncogenic properties of and their underlying mechanisms are shown in Figure 30.



**Figure 30.** Selected oncogenic properties of mutant p53 and their underlying mechanisms. The inner circle (shaded blue) represents oncogenic phenotypes associated with the activities of mutant p53 proteins. The outer circle depicts key mechanistic properties of p53 mutants that underlie the phenotypes listed in the inner circle. Note that each of the phenotypic effects can be attributed to almost each of the mechanistic properties; hence the inner blue circle can be freely rotated (Brosh and Rotter, 2009).

Inherited TP53 mutations are associated with a rare autosomal dominant disorder, the Li-Fraumeni syndrome (LFS). LFS is unusual because it causes susceptibility to a wide variety of cancers. It is characterized by multiple primary neoplasms in children and young adults, with a predominance of soft-tissue sarcomas, osteosarcomas, breast cancers, brain tumors and adrenocortical carcinomas (Li et al., 1988; Valdez et al., 2017). In about 70% of these multicancer families, mutant alleles of p53 were found to be transmitted in a mendelian fashion. Family members who inherited a mutant p53 allele had a high probability of developing some form of malignancy, often early in life. The age of onset of these various malignancies was found to be quite variable: about 5 years of age for adrenocortical



carcinomas, 16 years for sarcomas, 25 years for brain tumors, 37 years for breast cancer, and almost 50 years for lung cancer (Weinberg, 2013).

### **3.5.2 p53 and cancer treatment**

The TP53 gene is considered as one of the most important and well-known tumour suppressor gene and is the most frequently inactivated protein in human cancers, as it is mutated in 50% of human tumours (Leroy et al., 2014). Despite the high mutation rate of the protein p53, the remaining half of human tumours retains wild-type p53, implying that these tumours might have developed alternative mechanisms for disabling or attenuating specific p53 functions or the entire p53 pathway. This is also possible because p53 activity is strictly regulated, to avoid apoptosis induction in normal growth conditions. One of these regulatory mechanisms is represented by the ubiquitin-dependent degradation. Indeed, under non-stressed conditions, p53 is tightly regulated by the E3 ubiquitin ligase MDM2 protein through an auto-regulatory feedback loop, as this protein is also a p53 transcriptional target (Chene, 2003; Freedman et al., 1999; Picksley and Lane, 1993). Binding of MDM2 to the transactivation domain of p53 promotes the nuclear export of p53 and serves as a ubiquitin ligase that promotes ubiquitin-dependent degradation of p53 (Mendoza et al., 2014). The MDM2 gene has been found amplified or overexpressed in 7% of human malignancies, particularly in soft tissue tumours, testicular germ cell cancers and neuroblastoma (Oliner et al., 2016). It acts as an oncogene altering the p53 pathway especially in wild-type p53 tumour cells. It could then explain the resistance to cancer cell death in tumour cells expressing wild-type p53. Activation of p53 in human cancers harbouring wild-type p53 may offer a therapeutic benefit to induce cancer cell death. For this purpose, several small molecules able to inhibit p53-MDM2 binding have been developed, such as Nutlins, which activate the p53 pathway *in vitro* and *in vivo* (Vassilev et al., 2004). However, anti-tumour efficiency of p53 activation requires not only the presence of wild-type p53, but also functional p53 signalling pathway. Indeed, defects in the downstream p53 signalling decrease the activity of MDM2 inhibitors. For example, the MDM2 antagonist nutlin-3a is able to induce cell cycle arrest in several cancer-derived cell lines, but the apoptotic function of p53 is altered in some of them (Tovar et al., 2006).

Importantly, some other mechanisms have been developed in cancer cells to block apoptosis induced by p53, as the case for the couple Netrin-1/UNC5B, data in our lab strongly showing that an increase of Netrin-1 gene expression and a resistance to apoptosis upon p53 activation and induction (Paradisi et al., 2013). In addition, some papers also report that target to p53-dependent receptors-ligand complex, like UNC5B-Netrin-1 can also induce apoptosis dependent on the balance between the expression of receptor and ligand (Tanikawa et al., 2003).

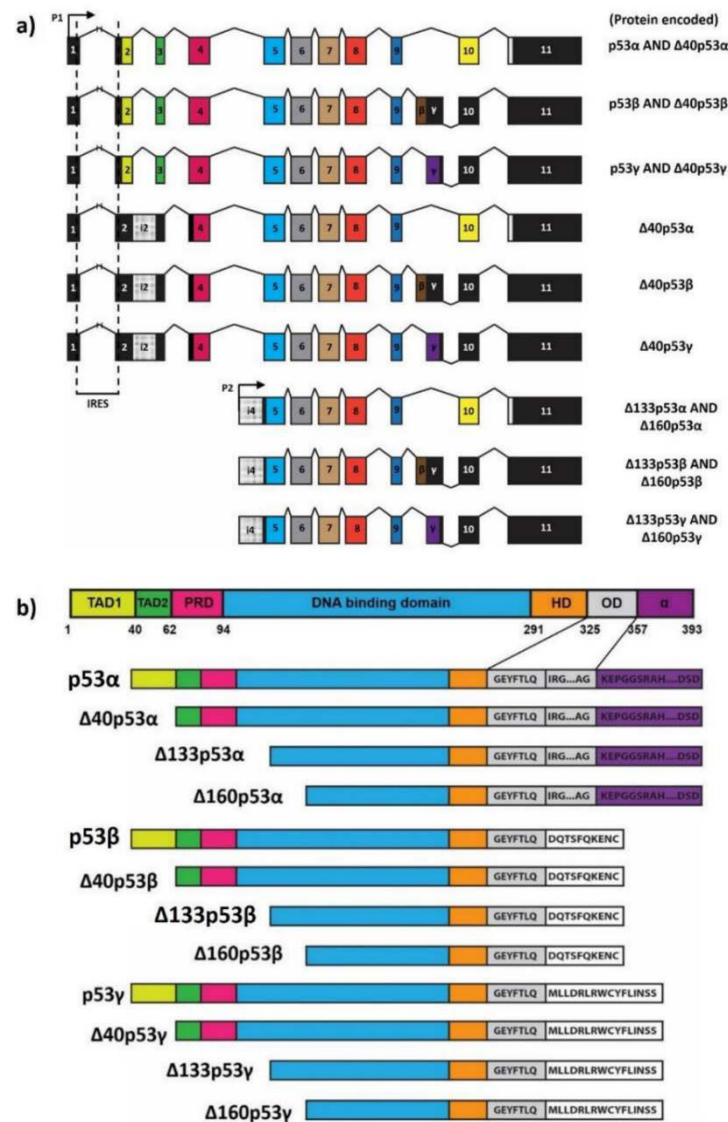
## 4. p53 isoforms

### 4.1 classification and structure in different species

In 1997, the two “younger siblings” of p53, p63 (Schmale and Bamberger, 1997) and p73 (Kaghad et al., 1997) were identified, displaying high homology to p53, for which reason these three proteins were defined to form the p53 family. After that, researchers found that p73 and p63 express multiple splice variants and N-terminally truncated forms (De Laurenzi et al., 1998; De Laurenzi et al., 1999; Yang et al., 1998). However, for years p53 was considered the only member of p53 family without splicing isoforms. The first observation of all p53 splice variants was reported in the 2005 by Jean-Christophe Bourdon et colleagues, and these isoforms were discovered in various species (Bourdon et al., 2005). However, existence of an N-terminal p53 isoforms was already described before (Courtois et al., 2002). Over the last decade, p53 isoform functions were hot studied, moreover, biological and clinical relevance have gradually been established (Joruz and Bourdon, 2016).

p53 isoforms are generated as a result of a combination of the use of alternative promoters, alternative splicing and alternative initiation of translation (Joruz and Bourdon, 2016). To date, it is reported that human TP53 differentially expresses in normal tissue with 12 mRNAs in a tissue-dependent manner. They are a result of alternative promoter usage and alternative splicing of intron-2 and intron-9. The alternative splicing of intron-9 of human TP53 produces three different C-terminal p53 forms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). Complete excision of intron-9 results in the expression of the  $\alpha$ -forms corresponding to the classical p53 C-terminal domain (oligomerization domain, OD). On the other hand, partial retention of intron-9 generates the  $\beta$ -or  $\gamma$ -forms, in which the OD is replaced by 10 or 15 new amino acids, respectively. The p53 mRNA transcribed from P1 promoter with spliced-out intron-2 can be translated from the first AUG (present in exon 2) and from AUG40 due to an internal ribosome entry site in the 5'UTR from exon1-exon2. The proteins translated from the first AUG generate p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ , and from AUG40 leads to the expression of  $\Delta 40$ p53 $\alpha$ ,  $\Delta 40$ p53 $\beta$ ,  $\Delta 40$ p53 $\gamma$  isoforms (Joruz and Bourdon, 2016). Transcription of p53 mRNA can also initiate from internal promoter P2,

which located at intron 4. Translation of these transcripts from initiating codons 133 and 160 can generate  $\Delta 133p53$  and  $\Delta 160p53$  isoforms, respectively, inducing  $\Delta 133p53\alpha$ ,  $\Delta 133p53\beta$ ,  $\Delta 133p53\gamma$ ,  $\Delta 160p53\alpha$ ,  $\Delta 160p53\beta$  and  $\Delta 160p53\gamma$  isoforms. Taken above together, in human, there are 12 p53 isoforms described: p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ ,  $\Delta 40p53\alpha$ ,  $\Delta 40p53\beta$ ,  $\Delta 40p53\gamma$ ,  $\Delta 133p53\alpha$ ,  $\Delta 133p53\beta$ ,  $\Delta 133p53\gamma$ ,  $\Delta 160p53\alpha$ ,  $\Delta 160p53\beta$ ,  $\Delta 160p53\gamma$  (Figure 31) (Anbarasan and Bourdon, 2019).



**Figure 31.** p53 mRNAs and functional domains of p53 and its isoforms.

**a)** p53 mRNAs. **b)** Functional domains of p53 and its isoforms. p53 contains seven functional domains which are the transactivation domain 1 (TAD1), the transactivation domain 2 (TAD2), the proline-rich domain (PRD), the DNA binding domain (DBD), the hinge domain (HD), the oligomerization domain (OD) and the negative regulation domain ( $\alpha$ ). At its N-terminus, lies an intrinsic disorder region (IDR) consisting of two acidic trans-activation domains (TAD), TAD1 (residues 1–39) and TAD2 (residues 40–61) and a proline-rich domain (PRD) (residues 62–93). This is followed by a DNA-binding domain (DBD) (residues 94–290) and a hinge domain (HD) (residues 291–324). At its carboxyl terminus, p53 comprises an oligomerization domain (OD) (residues 325–356) and a negative regulation domain ( $\alpha$ ) (residues 357–393) (Anbarasan and Bourdon, 2019).

p53 also has a complex gene structure in several other species such as mouse, zebrafish and drosophila. In mouse Mp53AS protein, the last 26 amino acids of Mp53 $\alpha$  are substituted by 17 others encoded by a cryptic exon in intron 10. Mp53AS protein is homologous to human  $\Delta$ 41p53 produced by alternative splicing of intron 2 is homologous to human  $\Delta$ 40p53 $\alpha$ . The Mp53 $\Psi$  is produced by alternative splicing of intron 6 adding 21 new amino acids. Up to now, no homologous protein to Mp53 has been found in other organisms or in human cells expressing the WT TP53 gene (Kazantseva et al., 2018). In zebrafish, Zp53 gene encodes Zp53 $\alpha$ , Zp53 $\beta$ , Z $\Delta$ Np53 $\alpha$  and Z $\Delta$ 113p53 $\alpha$  isoforms. In the Z $\Delta$ Np53 isoform, the first 33 amino acids are encoded by a cryptic exon in intron 2. In Zp53 $\beta$ , the last 19 amino acids are encoded by a cryptic exon in intron 8. Zp53 $\beta$  is not homologous to human p53 $\beta$  (Joruz and Bourdon, 2016). In drosophila, D $\Delta$ Np53 (also named Dp53) protein is encoded by an mRNA transcribed from an internal promoter. D $\Delta$ Np53 is homologous to  $\Delta$ 40p53/ $\Delta$ 133p53/ $\Delta$ Np63/ $\Delta$ Np73 proteins. Only DTAp53 protein contains the conserved box I (FxxLW) corresponding to the TA-1 of the p53 protein family (Bourdon et al., 2005) (Figure 32).

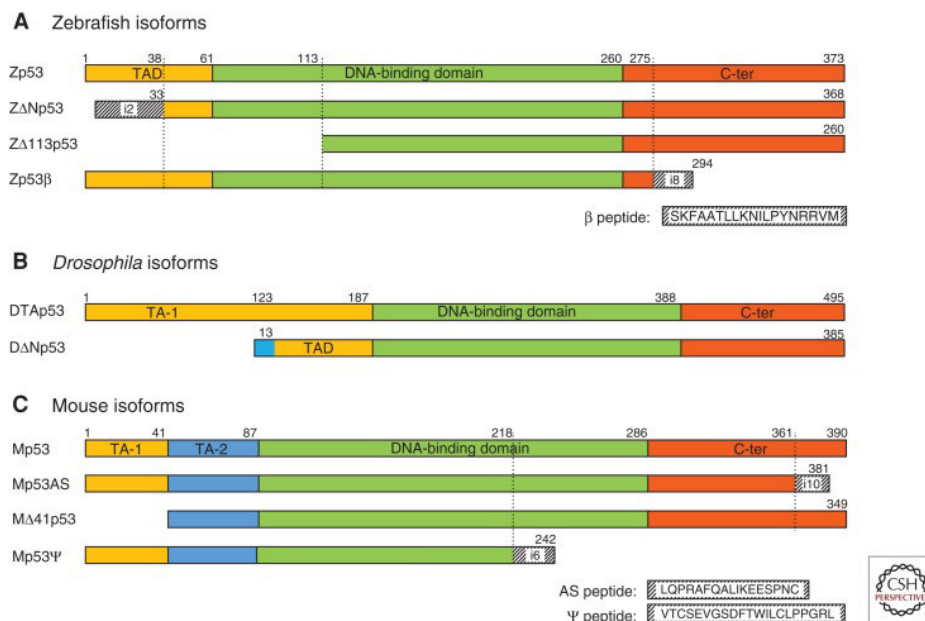


Figure 32. Scheme of p53 isoforms in zebrafish, drosophila and mouse (Joruz and Bourdon, 2016).

## 4.2 $\Delta$ 40p53 isoforms

As we have described in p53 chapter, human p53 protein contains two TADs, a PRR, a DBD, a TET and CT. The  $\Delta 40p53$  isoforms, also called  $\Delta N$ -p53 or p53/47, start from the codon 40 of the p53 mRNA, resulting in a shorter truncation. It has been detected in normal cells of human and mouse origin (Bourdon et al., 2005).  $\Delta 40p53$  could be generated from promoter P1 in two ways: from the same mRNA of full length p53, spliced for intron-2 and then from the use of an alternative IRES to encode  $\Delta 40p53$  from ATG in position 40; or from a different mRNA, retaining intron-2. In this latter case, as intron-2 carries an in-frame stop codon, the mRNA can produce only  $\Delta 40p53$  isoforms, using IRES just after stop codon, consequently restarting translation from codon 40 (Joruz and Bourdon, 2016). Considering  $\Delta 40p53$  isoforms lack TAD 1, but retains TAD 2, deepen understanding of the differences, the relationship (between two TADs) and biological functions of two TADs can help us better understand  $\Delta 40p53$  isoforms.

#### **4.2.1 Different functions of p53 TADs**

p53 is a well-known tumour suppressor gene, through its ability to regulate expression of different genes, involved in disparate cellular responses, including apoptosis, cell cycle arrest, senescence and metabolism (Vousden and Prives, 2009). p53 functions mainly depend by its ability to induce or repress transcription of target genes. However, transcription-independent activities of p53 has been described (Green and Kroemer, 2009; Moll et al., 2005). Transactivation activity of p53 requires two distinct TADs, comprising between residues 1-40 (TAD1) and 41-83 (TAD2) (Raj and Attardi, 2017). Reyes Candau and colleagues observed that TAD1 and TAD2 are able to work independently one to each other, with a similar transactivation activity, and TAD1 presents a slightly more activity than TAD2 (Candau et al., 1997). Moreover, recent work by the Attardi group, demonstrated two TADs functional specialization, the results indicating each TAD is required for transactivation of different target genes and effector pathways (Brady et al., 2011; Sullivan et al., 2018), this gives strong evidence of different functions of p53 two TADs.

Mutagenesis and reporter studies are used to distinguish the different functions of p53 TADs and specific residues are required for transcriptional activity within each

TAD. In TAD1, specific hydrophobic residues, Leu-22 (L22) and Trp-23 (W23) in human p53, are required for transcriptional activity, L22 and W23 must both be changed by mutagenesis to significantly inhibit p53 TAD1 transcriptional activation (Lin et al., 1994). Regarding TAD2, it was reported that two consecutive hydrophobic residues Try-53(W53), Phe-54(F54), which are surrounded by acidic amino acids, were indispensable for TAD2 transcriptional activity (Chang et al., 1995). By transient transfection experiments using luciferase reporter constructs, *in vitro* data showing that mutants in L22 and W23 (M22/23) induced a low but significant expression of reporter gene BAX, MDM2 and PIG3 (tumor protein p53 inducible protein 3) promoters, which are strongly correlated with p53-dependent apoptosis. This M22/23 mutant is not totally deficient but rather attenuated in its ability to induce p53-responsive apoptotic genes (Venot et al., 1999). Some paper reported M22/23 double mutant retains growth suppression and apoptotic activities in tumor cells, and these remaining activities could be due to TAD2 activity (Chen et al., 1996; Haupt et al., 1995). However, functional characterization of mutants in W53 and F54 (M53/54) revealed that it displays a phenotype which is intermediate to that displayed by p53 WT and M22/23. It exhibits slightly stronger growth suppression and apoptotic activities than M22/23. Mutation of both residues W53 and F54 together with L22 and W23 can generate quadruple mutant at positions 22, 23, 53 and 54 (p53<sup>QM</sup>), quadruple mutant p53<sup>QM</sup> totally lose growth suppression activity and this is correlated with a corresponding loss of apoptotic activity. More specifically, this mutant is completely deficient for BAX, MDM2, PIG3 and p21 transactivation promoters (Venot et al., 1999). *In vivo*, a series of TAD mutant knock-in mouse strains with alterations in the first (p53<sup>25,26</sup>), second (p53<sup>53,54</sup>), or both TADs (p53<sup>25,26,53,54</sup>), analogous mouse mutants, were generated. Results showing p53<sup>25,26</sup> is severely compromised for transactivation of most p53 target genes, and moreover, p53<sup>25,26</sup> cannot induce G1-arrest or apoptosis in response to acute DNA damage (Brady et al., 2011). Surprisingly, p53<sup>25,26</sup> retains robust activity in senescence and tumor suppression, indicating that efficient transactivation of the majority of known p53 targets is dispensable for these pathways. Both the p53<sup>25,26</sup> and p53<sup>53,54</sup> single TAD mutants retain the ability to drive senescence and tumor suppression, whereas p53<sup>25,26,53,54</sup> mutant cannot induce senescence or inhibit tumorigenesis, like p53 nullizygosity. In summary, *in vivo* and *in vitro* observations proved that TAD1 is critical for responses to acute

DNA damage and both TAD 1 and TAD 2 are responsible for mediating specific signaling pathways in tumor suppressive response of p53 (Table 4) (Figure 33) (Brady et al., 2011).

On embryonic development, because of p53-driven apoptosis or cell-cycle arrest in embryos, deleting either MDM2 or MDMX (a homologue of MDM2), in presence of full-length p53, results in embryonic lethality, at E5.5 or E7.5–11.5 respectively (Marine et al., 2006). As to the TAD mutants along with wild type p53, p53<sup>L25Q, W26S</sup> induce embryonic lethality at E10.5 because of neural tube closure defects, and p53<sup>F53Q, F54S</sup> mutant do not affect embryonic development, p53<sup>L25Q, W26S, F53Q, F54S</sup> induce lethality at E13.5, associated with phenotypes of coloboma, like fissure in the retina, or aberrant semicircular canal formation, and heart outflow tract defects. However, p53<sup>L25Q, W26S, F53Q, F54S/-</sup> can develop into viable adults, though they ultimately suffer with cancer. All the above indicate that mutant p53<sup>L25Q, W26S, F53Q, F54S</sup> have a genetic interaction with wild type p53 to induce these aberrant developmental phenotype, and transcriptional activation is vital for p53 to induce developmental defects (Johnson et al., 2005; Van Nostrand et al., 2014) (Table 4).

	transactivation	acute DNA damage responses	tumor suppression	embryonic lethality
P53 <sup>WT</sup>	+	+	+	-
P53 <sup>L25Q, W26S</sup>	+/-	-	+	+
P53 <sup>F53Q, F54S</sup>	+	+	+	-
P53 <sup>L25Q, W26S, F53Q, F54S</sup>	-	-	-	-

**Table 4.** Mouse p53 TAD mutant relative to WT p53. + indicates wild-type activity and - indicates lack of activity. +/- indicates selective transactivation, with severely compromised activation of a majority of p53 target genes and intact transactivation of a small group of p53 target genes. Acute DNA-damage responses encompass cell-cycle arrest and apoptosis in response to DNA damage. Embryonic lethality is in the context of one mutant allele and one p53 null allele (Raj and Attardi, 2017).

Studies open the door for detailed analyses of the mechanisms of p53 target gene induction during responses to acute DNA damage and oncogenic signaling. Mutations in key p53 TAD residues likely compromise critical interactions

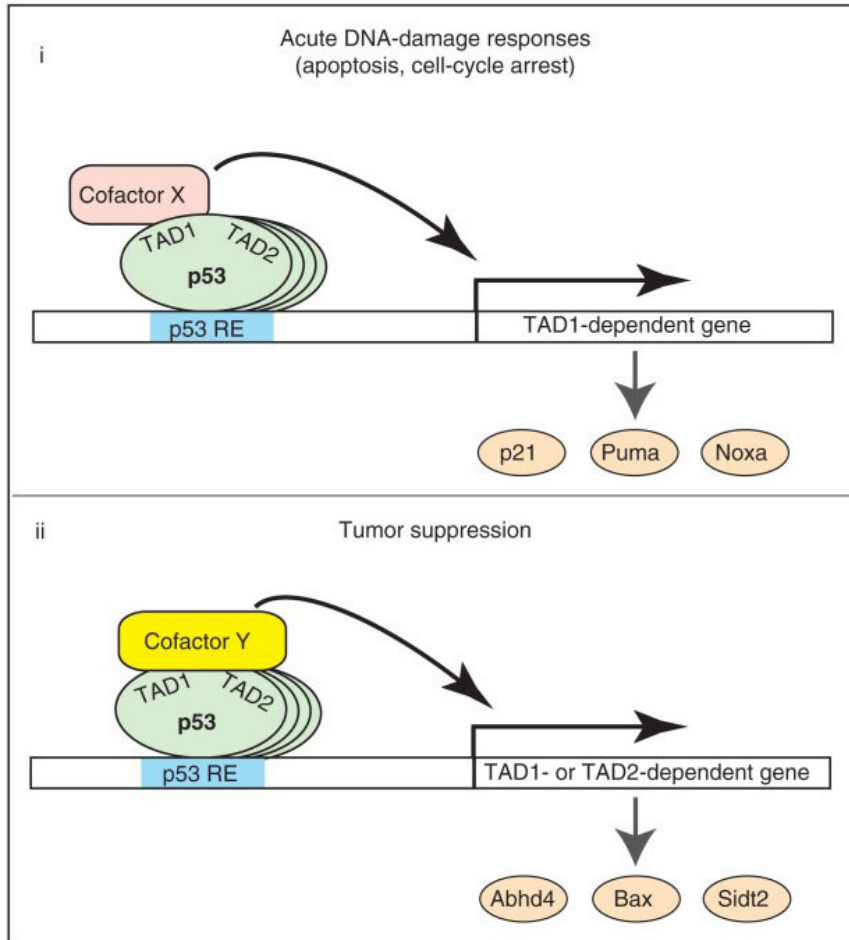


between p53 and important cofactors (Raj and Attardi, 2017). *In vitro* studies showing that mutation of TAD1 disrupts interactions with TBP, Taf9, Taf6, CBP, and the TRAP80 component of the mediator, mutation of TAD2 perturbs interaction with the p62 component of TFIIH, and mutation of both TADs impedes binding to p300 and STAGA. Although TAD I and TAD II are able to act independently, they also work in concert to recruit specific components of the multisubunit transcriptional activator STAGA complex, namely GCN5, Taf9, and ADA2b, in order to activate target genes such as p21, PUMA, and GADD45 (Raj and Attardi, 2017). Furthermore, the p53-mediated transcriptional repression that is induced by hypoxia requires both TAD I and TAD II (Table 5) (Figure 33).

<b>Interaction partner</b>	<b>Category</b>	<b>Interaction site on p53</b>	<b>References</b>
<b>Transcriptional machinery components</b>			
TBP	general transcription factors(GTFs)	TAD1	Seto et al. 1992; Chen et al. 1993b; Liu et al. 1993; Truant et al. 1993; Lin et al. 1994; Chang et al. 1995; Horikoshi et al. 1995
TAF6	GTFs	TAD1	Thut et al. 1995
TAF9	GTF	TAD1	Lu and Levine 1995; Thut et al. 1995
TFIIH (p62 and Tfb1)	GTF	TAD2	Xiao et al. 1994; Di Lello et al. 2006; Okuda and Nishimura 2014
Mediator (Med17)	Coactivator	TAD1	Ito et al. 1999; Meyer et al. 2010
<b>chromatin modifiers</b>			
p300/CBP	Histone acetyltransferases (HATs)	TAD1 and TAD2	Avantaggiati et al. 1997; Gu et al. 1997; Lill et al. 1997; Scolnick et al. 1997; Teufel et al. 2007; Feng et al. 2009; Lee et al. 2009, 2010a,b; Miller Jenkins et al. 2015

GCN5	HATs	TAD2	Gamper and Roeder 2008
PRMT1	Arginine methyltransferase	TAD1	An et al. 2004
<b>cellular processes of DNA replications, recombination, and repair</b>			
PC4	DNA metabolism and transcription	TAD2	Rajagopalan et al. 2009
RPA (RPA70N)	DNA metabolism and transcription	TAD2	Dutta et al. 1993; He et al. 1993; Li and Botchan 1993; Bochkareva et al. 2005
HMGB1	DNA metabolism and transcription	TAD2	Rowell et al. 2012
<b>P53 inhibitor</b>			
MDM2	E3 ubiquitin ligase	TAD1 and TAD2	Momand et al. 1992; Oliner et al. 1992; Chen et al. 1993a; Lin et al. 1994; Kussie et al. 1996; Blommers et al. 1997
MDMX	RING domain protein	TAD1 and TAD2	Shvarts et al. 1996; Hu et al. 2006; Popowicz et al. 2008
E1B	Adenoviral oncoprotein	TAD1	Lin et al. 1994

**Table 5.** p53 TADs interact with proteins involved in different steps of transcription (Raj and Attardi, 2017).



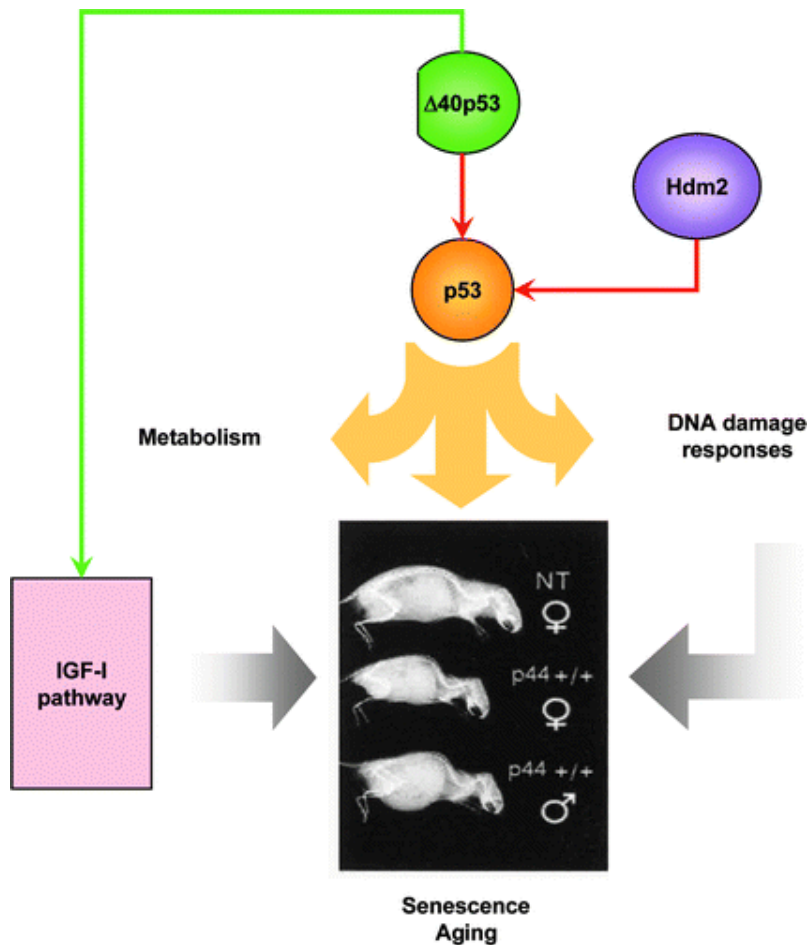
**Figure 33.** Models for p53 action in responses to acute DNA damage versus tumor suppression. TAD1 activity is required for transactivation of p53 target genes, such as p21, Puma, and Noxa, that mediate p53 DNA-damage responses, including apoptosis and cell-cycle arrest (i) TAD1 is dispensable for activation of some p53 target genes, such as Abhd4, Bax, and Sidt2, and for tumor suppression, as either TAD1 or TAD2 suffices for these responses. (ii) In model i, p53 uses TAD1 to interact with cofactor X to drive expression of TAD1-dependent genes. In model ii, p53 uses both TAD1 and TAD2 to interact with cofactor Y to activate genes associated with tumor suppression, as based on the observation that mutation of either TAD alone does not compromise expression of these genes.

#### 4.2.2 $\Delta 40$ p53 isoforms - N-terminal truncations of p53

As we mentioned before,  $\Delta 40$ p53 isoforms start from the codon 40 of the TP53 gene, lacking TAD 1, but retaining TAD 2 (Courtois et al., 2002). Hilla Solomon and colleagues discovered the generation of  $\Delta 40$ p53 isoform by proteasome and proved  $\Delta 40$ p53 are more stable than the p53, because  $\Delta 40$ p53 lacks of TAD1, preventing  $\Delta 40$ p53 degradation by MDM2 ((Nie et al., 2007; Pehar et al., 2014; Solomon et al., 2017).

Initially,  $\Delta 40p53$  were shown to oligomerize with full length p53 and negatively regulate the transcription and growth suppression (Courtois et al., 2002). Recently, more papers reported that  $\Delta 40p53$  have unique transcriptional targets and functions, and  $\Delta 40p53$  either alone or in combination with p53 is able to induce apoptosis. Indeed, biological functions of  $\Delta 40p53$  is influenced by the expression of other isoforms, for examples p53 $\alpha$ . As p53 forms a tetramer upon binding DNA, the interactions between p53 isoforms can be homo- or hetero-oligomers, thus directly conveying message and translating signaling in cell response. Hetero-oligomers  $\Delta 40p53\alpha/p53\alpha$  can modulate the transcriptional activity of IGF1-receptor and Nanog promoters, finally controlling the switch from pluripotent embryonic stem cells (ESCs) to differentiated somatic cells (Figure 35, left). Moreover,  $\Delta 40p53$  was discovered highly expressed in ESCs, and it is the major p53 isoform during early stages of embryogenesis in the mouse (Ungewitter and Scrable, 2010). In melanoma cells, exogenous  $\Delta 40p53$  induces apoptosis and increases expression of endogenous activated p53 $\alpha$ , forming  $\Delta 40p53\alpha/p53\alpha$  nuclear hetero-tetramers and modifying downstream p53 transcription target gene promoter activity, like LRDD (p53-induced protein with a death domain) and p21 genes, finally shifting cell-fate towards apoptosis and away from cell-cycle arrest (Takahashi et al., 2014).

There is strong evidence for biological role of  $\Delta 40p53$  in aging and senescence. Maier and colleagues have generated transgenic mice expressing  $\Delta 40p53$  (Maier et al., 2004). These mice show a profoundly changed growth rate, with a short size of body, and weight of many organs. Moreover, they present a premature aging phenotype with typical lordokyphosis and accelerated decrease in bone density, and this phenotype was not observed when the transgene was expressed in a p53-null mouse background. Mice lacking p53 are prone to multiple early cancers and expression of  $\Delta 40p53$  did not rescue that tumor-prone phenotype. In summary, the striking growth retardation and accelerated aging induced by  $\Delta 40p53$  was strictly dependent on the presence of p53. Moreover,  $\Delta 40p53$  transgenic mice are found altered, hyperactive IGF-I signaling pathway (Hafsi and Hainaut, 2011; Maier et al., 2004) (Figure 34).



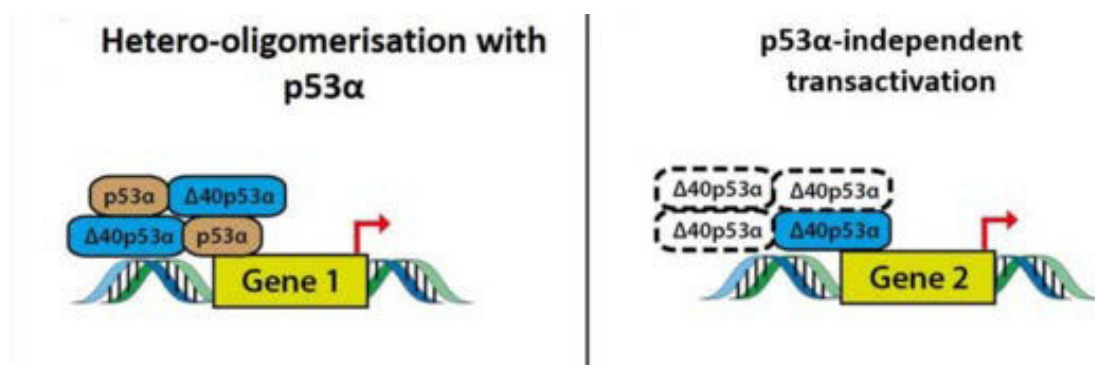
**Figure 34.** Role of  $\Delta 40p53$  in regulating aging and metabolism. Recent model proposes that  $\Delta 40p53$  may have a direct effect on the IGF-I signaling pathway, thus modulating important growth and survival factors in many tissues.  $\Delta 40p53$  may act as a regulator of p53 function by binding to the protein and affecting two of its properties, (1) its capacity to transactivate target genes, and (2) its capacity to bind MDM2 and to undergo proteasome-mediated degradation (Hafsi and Hainaut, 2011). The lower panel, showing radiological analysis of senescent mice, is taken from Maier et al., 2004 (Maier et al., 2004).

Recently, another paper, describing mice homozygous for a transgene encoding p44, shows cognitive decline and synaptic impairment early in life (Pehar et al., 2010). The synaptic deficits are attributed to hyperactivation of insulin-like growth factor 1 receptor (IGF1R) signaling and alterations in metabolism of the microtubule-binding protein tau. They were rescued by either IGF1 or MAPT (microtubule-associated protein tau) haploinsufficiency. When expressing a ‘humanized’ form of the amyloid precursor protein, p44<sup>+/+</sup> animals developed a selective degeneration of memory-forming and -retrieving areas of the brain, and died prematurely. The neurodegeneration was caused by both paraptosis- and autophagy-like cell deaths. In one word, altered longevity-assurance activity of p53:

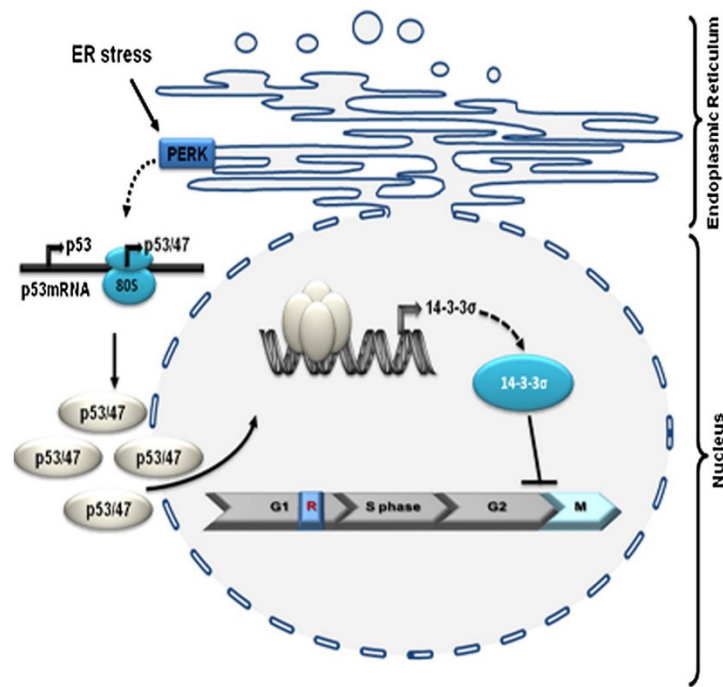
p44 causes memory loss and neurodegeneration by affecting IGF-1R signaling (Pehar et al., 2010).

$\Delta 40p53$  has also been described as a positive regulator of p53 function. For example, the biological function of hetero-oligomerization of isoforms are dose-dependent:  $\Delta 40p53\alpha$  at low levels increased p53 transactivation activity, on the contrary,  $\Delta 40p53\alpha$  at high levels inhibited p53 anti-proliferative effects (Hafsi et al., 2013). This means that p53 biological function is influenced by the relative ratio of different isoforms expression.

More interesting, in addition to forming  $\Delta 40p53/p53$  hetero-oligomers,  $\Delta 40p53$  can also regulate gene transcription independently from full-length p53. For example,  $\Delta 40p53\alpha$  can transactivate several p53 target genes, such as BAX and GADD45, in p53-null cells (Figure 35, right) (Yin et al., 2002). Other full-length p53-independent function of  $\Delta 40p53$  is induction of G2 arrest. It has been shown that full-length p53 controls the G1 phase of the cell cycle, while  $\Delta 40p53$  controls G2 progression (Vieler and Sanyal, 2018). Endoplasmic reticulum stress promotes PERK-dependent induction of  $\Delta 40p53$  mRNA translation and  $\Delta 40p53$  homo-oligomerization to transcriptionally activate targets 14-3-3 $\sigma$ , which targets CDC25, and thereby prevents the activation of the cyclin B/CDK1 complex, and results in a G2/M arrest (Bourougaa et al., 2010) (Figure 36).



**Figure 35.** Schematic of  $\Delta 40p53\alpha$  isoform two interactions way mediating transactivation (Anbarasan and Bourdon, 2019).



**Figure 36** Endoplasmic reticulum (ER) stress promotes PERK-dependent induction of  $\Delta 40p53$  mRNA translation and  $\Delta 40p53$  homo-oligomerization.  $\Delta 40p53$  induces 14-3-3 $\sigma$  and G2 arrest but does not affect G1 progression (Bourougaa et al., 2010).

Initial studies suggested that  $\Delta 40p53$  had no activity on its own, and subsequently several studies showing  $\Delta 40p53$  acted as a dominant-negative inhibitor of p53-mediated transactivation, growth suppression and apoptosis. Now it is now apparent that  $\Delta 40p53$  functions are far more complex.  $\Delta 40p53$  has been shown to change p53 target gene expression in both a positive and negative way to modulate the biological outcome of WT p53 activation. In addition,  $\Delta 40p53\alpha$  in its own can also independently mediate transactivation process (Vieler and Sanyal, 2018; Yin et al., 2002). The functional impact of  $\Delta 40p53$  related to its relative levels and on the cellular context.

### 4.2.3 $\Delta 40p53$ and cancer

Although many aspects of p53 in cancer have been thoroughly investigated, the role and regulation of p53 isoforms remain not well understood. Recent studies suggested transgenic expression  $\Delta 40p53$ , does not lead to tumor formation in mice, but is associated with a short life span, cognitive decline, and overt diabetes (Maier et al., 2004; Pehar et al., 2010). However, high expression of  $\Delta 40p53$  was reported in human melanoma cell lines and primary melanoma isolates (Avery-Kiejda et al.,

2008). Though several aspects of  $\Delta 40p53$  isoforms have already been discovered and reported, more studies are still needed to better understand the role of  $\Delta 40p53$  isoforms in different cancers.

#### **4.2.3.1 $\Delta 40p53$ is a major isoform in glioblastoma**

Takahashi and colleagues examined 17 glioblastoma xenograft tissues gliosis, non-tumor cortex, and neural progenitor cells for p53 isoform expression. Using mass spectrometry and several characterized p53 antibodies, they performed a comprehensive analysis of the p53 isoforms. Interestingly, results show that both adult subventricular zone progenitor cells and gliotic brain tissue undergo proliferation at low rates, and in response to injury, share the expression of  $\Delta 40p53$  in the absence of full-length p53. The isoform profile of glioblastoma xenografts finds  $\Delta 40p53$  as a major isoform, resembling to highly proliferative, undifferentiated stem cells. However, differentiated subventricular zone progenitor cells and tissue from normal brain express low to undetectable levels of  $\Delta 40p53$  and no p53. In summary,  $\Delta 40p53$ , as neural progenitor marker, is increased in the astrocytic response to injury and in glioblastoma, both of them have the capacity of proliferation but not in cerebral cortex that is primarily composed of terminally differentiated cells or in the differentiated progeny subventricular zone progenitor cells (Takahashi et al., 2013).

#### **4.2.3.2 $\Delta 40p53$ is the highest expressed p53 isoform in breast cancers**

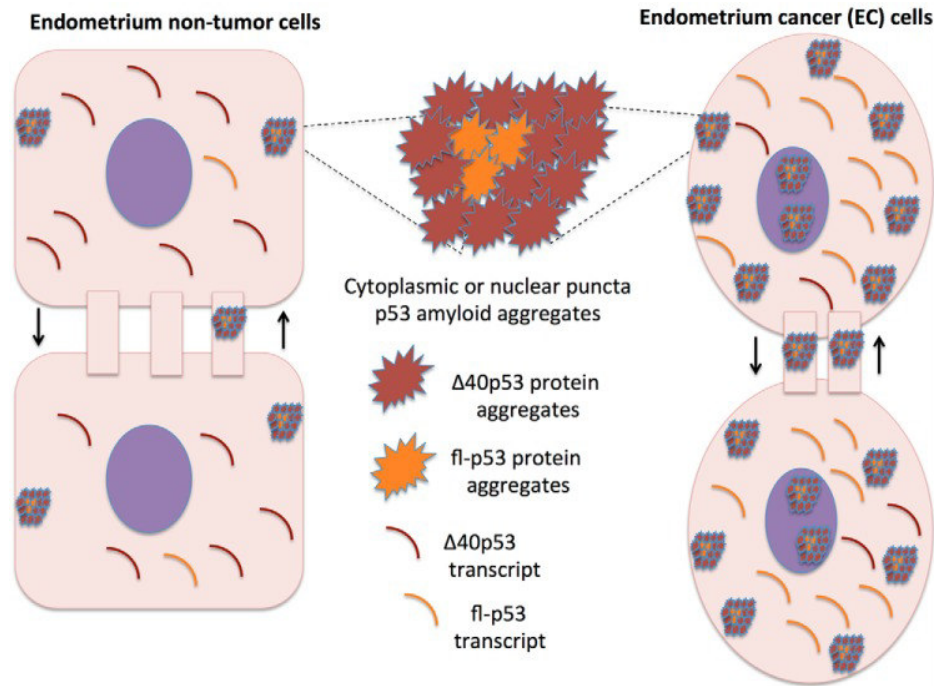
Breast cancer is the most common malignancy developing in women, but somatic mutations of p53 are rare, suggesting that p53 becomes inactivated by other mechanisms. Avery-Kiejda and colleagues have analyzed the relative mRNA expression of the p53 isoforms,  $\Delta 40p53$ ,  $\Delta 133p53$ , p53 $\beta$  and p53 $\gamma$  in a panel of 6 breast cancer cell lines, including 31 matched normal adjacent tissues as well as 148 breast cancers specimens and 31 matched normal adjacent tissues by semi-quantitative real-time reverse transcription-PCR and analyzed their relationship to clinical features and outcome. They identified several important clinical associations, particularly with  $\Delta 40p53$ , which was expressed at levels that were



~50-fold higher than the least expressed isoform p53 $\gamma$ .  $\Delta$ 40p53 was significantly upregulated in tumor tissue when compared with the normal breast and was significantly associated with an aggressive breast cancer subtype-triple negative (Avery-Kiejda et al., 2014). In another study, RNA levels from breast cancer specimens were analyzed by using the hybridization-based QuantiGene 2.0 Assay, demonstrating that  $\Delta$ 40p53 is the most highly expressed p53 isoform in a cohort of 148 fresh frozen breast tumor tissues (Avery-Kiejda et al., 2014). Full-length p53 mRNA expression was detected using qPCR in both FFPE and FF tissues, however,  $\Delta$ 40p53 mRNA can only be detected in fresh frozen tissues (Morten et al., 2016). Data shown above leads to the conclusion that  $\Delta$ 40p53 is selectively increased in breast cancer, and strongly associated with an aggressive breast cancer subtype-triple negative.

#### **4.2.3.3 $\Delta$ 40p53 and p53 amyloid aggregate formation in endometrial carcinoma**

Endometrial carcinoma (EC) is a type of cancer in which p53 status is related with prognosis. It has been shown that full-length p53 and  $\Delta$ 40p53 isoform are the predominantly expressed p53 variants in endometrial carcinoma cells, while  $\Delta$ 40p53 was the major p53 isoform in endometrial nontumor cells (Melo Dos Santos et al., 2019).  $\Delta$ 40p53 is mainly localized to cytoplasmic punctate structures of endometrial carcinoma cells, resembling solid-phase structures similar to the ones found in neurodegenerative pathologies. Using light-scattering kinetics, circular dichroism, and transmission electron microscopy, researchers noted that the p53 N-terminal transactivation domain significantly reduces aggregation of the WT p53 DNA-binding domain, confirming the higher aggregation tendency of  $\Delta$ 40p53, which lacks this domain. It is the first report of cytoplasmic  $\Delta$ 40p53 in EC cells being a major component of amyloid aggregates. The differential aggregation properties of p53 isoforms in EC cells may open up new avenues in the development of therapeutic strategies that preferentially target specific p53 isoforms to prevent p53 amyloid aggregate formation (Melo Dos Santos et al., 2019) (Figure 37).



**Figure 37** The  $\Delta 40p53$  isoform in EC cells.

The schematic shows  $\Delta 40p53$  as a modulator of p53 tumor suppression and oncogenic activities in endometrial carcinoma cells. Although the full-length p53 transcript is the major expressed p53 transcript,  $\Delta 40p53$  is the predominant component of p53 amyloid aggregates in endometrial carcinoma cells. Once  $\Delta 40p53$  was the major component of p53 cytoplasmic aggregates, it will be a key modulator of p53 tumor suppression and oncogenic activities, same trend shown in melanoma, breast tumors and hepatocellular carcinoma, in which  $\Delta 40p53$  isoform presents a dominant-negative role over full-length p53.  $\Delta 40p53$  may be mainly sequestered in cytoplasmic amyloid aggregates, similar to those either modulate p53-mediated tumor suppressor roles or its oncogenic activities. The presence of these structures in cellular projections between adjacent cells suggests that they can be transported from cell to cell.  $\Delta 40p53$  may modulate p53 aggregation properties and endometrial carcinoma progression, in which p53 genetic alterations are related to a poor prognosis (Melo Dos Santos et al., 2019).

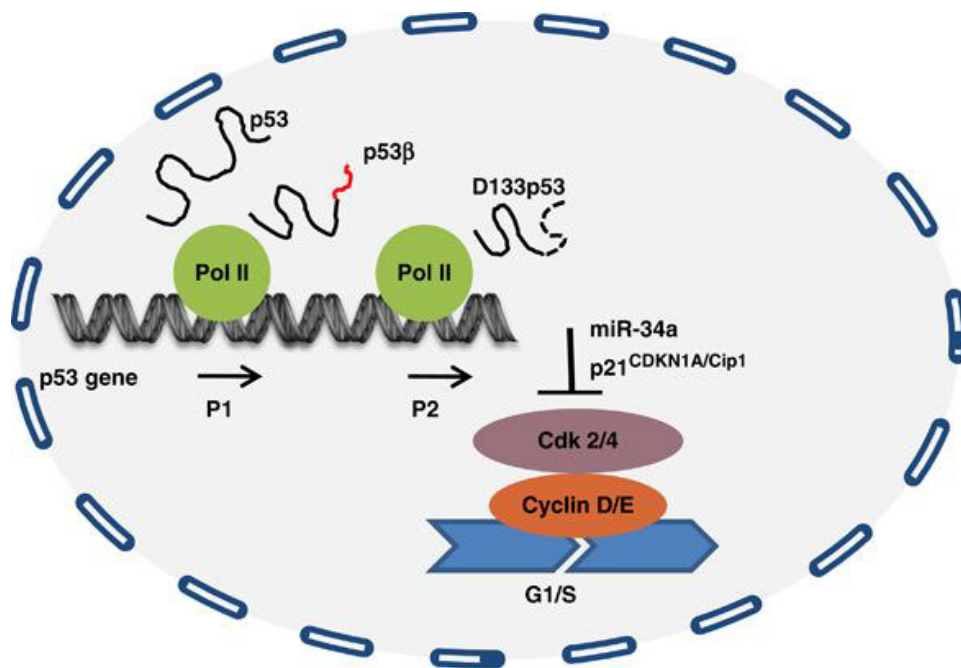
### 4.3 Role of $\Delta 133p53$ and $\Delta 160p53$ isoforms in cancer

TP53 mRNA gene transcription can be initiated from internal promoter which is located at intron 4, and translation of these transcripts from codon 133 produces  $\Delta 133p53$  isoform and codon 160 generates  $\Delta 160p53$  isoform. Considering intron 9 alternative splicing, promoter P2 gives rise to six isoforms ( $\Delta 133p53\alpha$ ,  $\Delta 133p53\beta$ ,  $\Delta 133p53\gamma$ ,  $\Delta 160p53\alpha$ ,  $\Delta 160p53\beta$  and  $\Delta 160p53\gamma$  isoforms) (Joruiz and Bourdon, 2016).  $\Delta 133p53$  isoforms lack a portion of first conserved cysteine box of the DBD, so it lacks DNA-binding capacity towards p53 response elements *in vitro* and counteracts the capacity of p53 to suppress cell growth. Then,  $\Delta 133p53$  can neutralize the suppressive activity of FLp53.

$\Delta 133p53$  isoforms can bind to p53-response elements to induce the p53 mediated response. Interactions between  $\Delta 133p53$  isoforms and target genes can be indirect to convey and translate cell signaling in cell response. For example, after exposed to  $\gamma$ -irradiation 24h, human colorectal carcinoma cells HCT116 accumulate high levels of both  $\Delta 133p53$  and p73 (Nutthasirikul et al., 2015). In p53-null H1299 cells, the increased DNA double-strand break repair was associated with the overexpression of  $\Delta 133p53\alpha$  isoforms and was reduced upon knockdown of p73. The changes in p73 alone had no effect of DNA double-strand break repair suggesting that p73 and  $\Delta 133p53\alpha$  isoforms can cooperate to upregulate the transcription of DNA double-strand break repair genes RAD51, RAD52 and LIG4 (Gong et al., 2018; Gong et al., 2015).  $\Delta 133p53$  isoforms also associated with the small GTPase RhoB (an anti-apoptotic protein). It has been shown that by inhibiting RhoB activity,  $\Delta 133p53$  protects cells from camptothecin-induced apoptosis, and high  $\Delta 133p53$  mRNA expression levels are correlated with higher risk of recurrence in patients with locally advanced rectal cancer, suggesting that  $\Delta 133p53$  can act as an oncogene (Arsic et al., 2017).

Moreover,  $\Delta 133p53$  exerts its role by modulating full-length p53 signaling to extend the replicative lifespan and promotes spontaneous DNA double strand breaks repair.  $\Delta 133p53$  dominant-negative inhibition of FLp53 occurs directly at two p53-senescence associated genes promoters: p21 and miR34a, suggesting that  $\Delta 133p53$  modulates p53 signaling to repress early onset of senescence in Hutchinson-Gilford Progeria Syndrome cells (von Muhlinen et al., 2018). It has been also reported that  $\Delta 133p53$ , together with p53 $\beta$ , could regulate cellular senescence (Mondal et al., 2013).  $\Delta 133p53$  lacks TADs and suppresses p53 transaction and the expression of pro-senescence factors p21 and miR-34a, p21 binds to and inactivates the G1/S cyclin–CDK complexes, and miR-34a targets cyclin E and CDK4 expression. On the other hand, p53 $\beta$  cooperates with FLp53 to promote p21 expression. The combined effect of more p53 $\beta$  and less  $\Delta 133p53$  is related with the induction of senescence (Figure 38) (Olivares-Illana and Fahraeus, 2010). The dominant-negative effect of  $\Delta 133p53\alpha$  on p53 $\alpha$  is not universally, it is context and DNA-sequence dependent. For example, in U2OS cells, the induction

of  $\Delta 133p53\alpha$  inhibited p53 $\alpha$ -dependent apoptosis and G1 arrest but not p53 $\alpha$ -dependent G2 arrest (Aoubala et al., 2011).



**Figure 38** p53 isoforms ( $\Delta 133p53$ , p53 $\beta$ ) activation under certain conditions can specifically result in senescence (von Muhlinen et al., 2018).

$\Delta 160p53\alpha$  can be detected in several cell lines, including K562, despite the presence of a pre-mature stop at codon 148. K562 cells express C-terminal variant  $\Delta 160p53\beta$ , which is repressed by hemin treatment (Marcel et al., 2010). Marco M Candeias and colleagues found most common p53 cancer mutants express a larger number and higher levels of shorter p53 isoforms that are translated from the mutated FLp53 mRNA. Cells expressing mutant p53 exhibit gain-of-function cancer phenotypes, such as enhanced proliferation, cell survival, invasion and adhesion, changed mammary tissue architecture and invasive cell structures. Interestingly,  $\Delta 160p53$  overexpressing cells behave in a similar manner. However exogenous or endogenous mutant p53 that fails to express  $\Delta 160p53$  because of specific mutations or antisense knockdown loses its pro-oncogenic potential (Candeias et al., 2016).

## 4.4 Scientific tools to investigate p53 isoforms

### 4.4.1 (RT-q) PCR

The TP53 gene expresses different mRNA variants (p53 isoform mRNAs), due to alternative splicing and internal promoter usage. The dual promoter structure of the TP53 gene results in the production of two subclasses of mRNA variants which differ in their 5'UTR (TAp53 and  $\Delta$ 133p53 subclasses). A third subclass of mRNA variant ( $\Delta$ 40p53 subclass) retains the entire intron 2 by alternative splicing. These three different subclasses can be combined to three distinct sub-classes of mRNA variants differing by their 3'UTR due to alternative splicing of intron 9 ( $\alpha$ ,  $\beta$ , and  $\gamma$  subclasses) (Bourdon et al., 2005). p53 mRNA variants have distinct nucleotide sequences allowing the design of primers and probes specific for p53 isoforms. Up to now, the most reliable method to identify and quantify p53 splice variants is quantitative reverse transcription polymerase chain reaction or quantitative real-time RT-PCR (Khoury et al., 2013). RT-qPCR enables the quantification of subclasses of p53 mRNA variants (i.e., TAp53,  $\Delta$ 40p53,  $\Delta$ 133p53,  $\alpha$ ,  $\beta$ , and  $\gamma$  subclasses) (Figure 39). However, there is not always a strict correlation between the p53 mRNA variants expression and p53 protein isoforms because p53 protein isoforms are also regulated at the posttranscriptional level.

p53 isoform subclass	Primer name	Targeted region	5'-3' sequence
TA/ $\Delta$ 40p53	(F)	Exon 4	CAGCCAAGTCTGTGACTTGCA
	(R)	Exon 5	GTGTGGAATCAACCCACAGCT
	(Pr)	Exon 5	TCCCCTGCCCTCAACAAG ATGTTTTGCC
$\Delta$ 133p53	(F)	Intron 4	ACTCTGTCTCCTTCCTTCTCCTACAG
	(R)	Exon 5	Same as TAp53 (R)
	(Pr)	Exon 5	Same as TAp53 (Pr)
$\alpha$ (alpha)	(F)	Exon 9	AACCACTGGATGGAGAATATTTTAC
	(R)	Exon 10	CAGCTCTCGGAACATCTCGAA
	(Pr)	(Exon 9/10)	TCAGATCCGTGGGCGTGAGCG
$\beta$ (beta)	(F)	Exon 9	Same as p53 $\alpha$ (F)
	(R)	(Exon 9 $\beta$ )	TCATAGAACCATTTTCATGCTCTCTT
	(Pr)	(Exon 9/exon 9 $\beta$ )	CAGGACCAGACCAGCTTTCAAAAAGAAAATTGTT
$\gamma$ (gamma)	(F)	Exon 9	Same as p53 $\alpha$ (F)
	(R)	(Exon 9 $\gamma$ )	TCAACTTACGACGAGTTTATCAGGAA
	(Pr)	(Exon 9/exon 9 $\gamma$ )	TTCAGATGCTACTTGACTTACGATGG
E8/9	(F)	Exon 8	GAAGAGAATCTCCGCAAGAAAGG
	(R)	Exon 9	TCCATCCAGTGGTTTCTTCTTTG
	(Pr)	Exon 8/9	AGCACTAAGCGAGCACTGCCCAACA

**Figure 39.** Primers and probes for quantitative amplification of human p53 isoforms by RT-qPCR (Real-Time PCR using the TaqMan® chemistry)

#### 4.4.2 small Interfering RNAs (siRNAs)

Jean-Christophe Bourdon and other scientists have already developed several siRNAs and shRNAs targeting specifically distinct exons or introns of human TP53, which enable knock- down differentially and specifically subsets of human p53 splice variants. To discover the biological activities of endogenous p53, siRNAs are essential tools (Joruiz and Bourdon, 2016). Using unique sequences present in the different isoforms, it is possible to design siRNA, targeting specific p53 isoforms. These siRNA sequences are presented in the Table 6, together with targeted isoforms.

	Positionon sequence (nt) GenBank accession number (NG_017013)	Exon location	Exon sequence	Targeted forms
siE2	15968 15986	- Exon 2	GCAGUCAGAUCCUAGCGUC	p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ , $\Delta$ 40p53 $\alpha$ , $\Delta$ 40p53 $\beta$ , $\Delta$ 40p53 $\gamma$
si133a	17212 17230	- Intron 4	GGAGGUGCUUACACAUGUU	$\Delta$ 133p53 $\alpha$ , $\Delta$ 133p53 $\beta$ , $\Delta$ 133p53 $\gamma$ , $\Delta$ 160p53 $\alpha$ , $\Delta$ 160p53 $\beta$ , $\Delta$ 160p53 $\gamma$
si 133b	17271 17289	- Intron 4	CUUGUGCCCUGACUUUCA	$\Delta$ 133p53 $\alpha$ , $\Delta$ 133p53 $\beta$ , $\Delta$ 133p53 $\gamma$ , $\Delta$ 160p53 $\alpha$ , $\Delta$ 160p53 $\beta$ , $\Delta$ 160p53 $\gamma$
si E7	18322 18340	- Exon 7	GCAUGAACCGGAGGCCCAU	All
si E7/E8	18363 18370/ 18714 - 18724	- Exon 7 and 8	GACTCCAGTGGTAATCTAC	All
si $\beta$	19211 19229	- Exon 9 $\beta$	GGACCAGACCAGCUUUCA	p53 $\beta$ , $\Delta$ 40p53 $\beta$ ,

si $\gamma$	19009 19016/ 19285 - 19295	-	Exon 9 and $9\gamma$	CCCUUCAGAUGCACUUGA	$\Delta 133p53\beta$ , $\Delta 160p53\beta$ p53 $\gamma$ , $\Delta 40p53\gamma$ , $\Delta 133p53\gamma$ , $\Delta 160p53\gamma$
si Splice	19284 19302	-	Exon $9\beta$ and $9\gamma$	GAUGCACUUGACUUACGA	p53 $\beta$ , $\Delta 40p53\beta$ , $\Delta 133p53\beta$ , $\Delta 160p53\beta$ , p53 $\gamma$ , $\Delta 40p53\gamma$ , $\Delta 133p53\gamma$ , $\Delta 160p53\gamma$
si $\alpha$	21846 21864	-	Exon 10	GUGAGCGCUUCGAGAUGUU	p53 $\alpha$ , $\Delta 40p53\alpha$ , $\Delta 133p53\alpha$ , $\Delta 160p53\alpha$

**Table 6.** p53 isoform-specific small interfering RNAs (Joruiiz and Bourdon, 2016).

### 4.4.3 antibodies

Up to now, a series of antibodies are produced to detect different isoforms, for example, mouse monoclonal antibodies DO-1 and DO-7 are widely used, can recognize the same epitope within the TAD1 and can only detect p53 $\alpha$ , p53 $\beta$ , and p53 $\gamma$ . The mouse monoclonal antibody 1801 recognizes an epitope within the TAD2, so it detects p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ ,  $\Delta 40p53\alpha$ ,  $\Delta 40p53\beta$ , and  $\Delta 40p53\gamma$ . The mouse monoclonal 421 and BP53.10 recognize a similar epitope in the  $\alpha$  domain and, thus, detect p53 $\alpha$ ,  $\Delta 40p53\alpha$ ,  $\Delta 133p53\alpha$ , and  $\Delta 160p53\alpha$ . Mouse monoclonal antibodies DO-11, 240, DO-12, and 1620 can recognize different epitopes in the DBD, so theoretically they detect all p53 isoforms. The rabbit CM1 and sheep SAPU polyclonal antibodies have been raised against bacterially produced recombinant human p53 $\alpha$ , they recognize several distinct epitopes in the amino-terminal and  $\alpha$  domains so they detect p53 $\alpha$  strongly compared with the other p53 isoforms. CM1 and SAPU antibodies have a lower affinity for the  $\Delta 133/\Delta 160$  and  $\beta/\gamma$  forms that lack the amino terminus or c-terminus epitopes, respectively (Joruiiz and Bourdon, 2016) (Table 7).

Epitope				
	Amino acids	Exon	Sequence	Recognized forms
DO-1	20–25	2	SDLWKL	p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$
DO-7	20–25	2	SDLWKL	p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$
1801	46–55	4	SPDDIEQWFT	p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ , $\Delta$ 40p53 $\alpha$ , $\Delta$ 40p53 $\beta$ , $\Delta$ 40p53 $\gamma$
1620	145 – 157+ 201 – 212	5/6	LWVDST PPPGTRV + LRVEYLDDRN TF	ND
DO-11	181 – 190	5/6	RCSDSDGLAP	All
240	211 – 220	6	TFRHSVVVPY	All
DO-12	256 – 267	7/8	TLEDSSGNLLGR	All
SAPU	11–65/ 290 – 301/ 363 – 393	2/3/4/ 8/9/ 10/11	EPPLSQ- DPGPDEAPR/RKKG EPHHELPP/RAHSSH LKSKKGQSTSRHK KLMFKTEGPDSD	All
CM1	1–90/ 379 – 393	2/3/ 4/11	MEEPQSDPSV. . . . . TPAAPAPAPS/ RHKKLMFKTEGPD SD	p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ , $\Delta$ 40p53 $\alpha$ , $\Delta$ 40p53 $\beta$ , $\Delta$ 40p53 $\gamma$ , $\Delta$ 133p53 $\alpha$ , $\Delta$ 160p53 $\alpha$
421	372 – 379	11	KKGQSTSR	p53 $\alpha$ , $\Delta$ 40p53 $\alpha$ , $\Delta$ 133p53 $\alpha$ , $\Delta$ 160p53 $\alpha$
BP53. 10	374 – 378	11	GQSTS	p53 $\alpha$ , $\Delta$ 40p53 $\alpha$ , $\Delta$ 133p53 $\alpha$ , $\Delta$ 160p53 $\alpha$

**Table 7.** p53 isoform-specific antibodies (Joruiz and Bourdon, 2016).



## **II. Aims of Thesis Project**

Dependence receptors represent a new class of transmembrane receptors able to trigger to opposite pathways. Indeed, in presence of their ligand these receptors induce a “positive” signaling of differentiation and cell survival. However, opposite to most cellular receptors, they are also active in the absence of their ligands. In these settings, dependence receptors transduce a “negative” signaling that triggers caspase-dependent apoptotic cell death (Goldschneider and Mehlen, 2010). For this reason, these receptors have been dubbed “dependence receptors” since expression of such dependence receptor renders cells dependent on availability of the ligand for survival (Mehlen and Puisieux, 2006). Most of dependence receptors were proposed to act as tumor suppressors. The overall hypothesis is that expression of such dependence receptor could prevent tumor development by inducing the apoptosis of tumor cells that would proliferate in excess compared to the limiting amount of ligand in the milieu or migrate beyond the regions of ligand availability. As a consequence, cell transformation toward a malignant phenotype requires inactivation of dependence receptors, that could be achieved, between other ways, by up-regulation of ligand expression. This was demonstrated for the ligand Netrin-1 and its dependence receptors DCC and UNC5H family. Indeed, Netrin-1 is over-expressed in several aggressive cancers. In our laboratory, we have shown that in these human cancers expressing Netrin-1 it is possible to induce cell death and tumor regression by directly interfering with the binding between Netrin-1 and its receptors. To this purpose, we have developed a neutralizing Netrin-1 monoclonal antibody, called NP137, that was humanized and recently entered in phase I clinical trial at Léon Bérard cancer center in Lyon (Grandin et al., 2016b).

However, until now we have little information about the molecular mechanisms involved in Netrin-1 up-regulation in cancer cells. This knowledge could be important to increase the fraction of cancer patients who would be eligible to a Netrin-1 interference-based treatment during early clinical evaluation. Recently, we have shown that Netrin-1 gene expression is regulated by p53 (Paradisi et al., 2013). This protein is a master transcription factor regulating cellular processes such as apoptosis and cell cycle arrest. Several p53 splicing variants were identified in 2005 in different species and their biological and clinical relevance have been studied (Anbarasan and Bourdon, 2019). Till date, twelve protein isoforms of p53 (p53 $\alpha$ , p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 40p53 $\alpha$ ,  $\Delta$ 40p53 $\beta$ ,  $\Delta$ 40p53 $\gamma$ ,  $\Delta$ 133p53 $\alpha$ ,  $\Delta$ 133p53 $\beta$ ,

$\Delta 133p53\gamma$ ,  $\Delta 160p53\alpha$ ,  $\Delta 160p53\beta$ ,  $\Delta 160p53\gamma$ ) encoded by *TP53* mRNA transcripts, have been described and characterized in humans (Joruiz and Bourdon, 2016).

The main aim of our work, presented here, is to better characterize how p53 regulates Netrin-1 gene expression. In particular, we plan to (i) verify whether p53-dependent regulation of Netrin-1 requires transcriptional activity, generating several stable cell lines inducible for mutated p53 proteins, in order to invalidate transcriptional activity of both p53 transactivation domains; (ii) demonstrate that p53 isoforms, in particular N-terminal truncated proteins, are able to regulate Netrin-1 gene expression, in presence or not of full-length p53; (iii) assess if regulation of Netrin-1 gene expression by p53 isoforms could increase cell survival dependence to Netrin-1, by evaluating cell death induction following Netrin-1 inhibition; (iv) finally correlate Netrin-1 and p53 isoforms gene expression in different human cancer patients.

In conclusion, this information could increase the fraction of patients eligible to anti-Netrin-1 treatment. Indeed, analysis of p53 isoforms gene expression could allow a personalized therapy targeting Netrin-1 and reactivating dependence receptors-induced cell death and tumor regression.

### **III. Results**

**Article:  $\Delta$ 40p53 isoform regulates Netrin-1 and its dependence receptor  
UNC5B gene expression, enhancing tumour cell survival**  
(In submission)

# **$\Delta$ 40p53 isoform regulates Netrin-1 and its dependence receptor UNC5B gene expression, enhancing tumour cell survival**

Running title: Regulation of Netrin-1 expression by  $\Delta$ 40p53

Yan Sun<sup>1</sup>, Ambroise Manceau<sup>1\*</sup>, Lucie Cappuccio<sup>3\*</sup>, Valeria Basso<sup>1</sup>, Hong Wang<sup>1</sup>, Carine Maisse<sup>3</sup>, Patrick Mehlen<sup>1,2,4§</sup>, and Andrea Paradisi<sup>1§</sup>

<sup>1</sup>Apoptosis, Cancer and Development Laboratory - Equipe labellisée 'La Ligue', LabEx DEVweCAN, Centre de Recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Université Claude Bernard Lyon1, Centre Léon Bérard, 69008 Lyon, France.

<sup>2</sup>Netris Pharma, 69008 Lyon, France.

<sup>3</sup>IVPC UMR754 INRA, Université de Lyon, Université Claude Bernard Lyon1, EPHE, Lyon, France.

<sup>4</sup>Department of Translational Research and Innovation, Centre Léon Bérard, 69008 Lyon, France.

\*These authors contributed equally to this work

§Co-senior and Corresponding authors: [andrea.paradisi@lyon.unicancer.fr](mailto:andrea.paradisi@lyon.unicancer.fr) or [patrick.mehlen@lyon.unicancer.fr](mailto:patrick.mehlen@lyon.unicancer.fr).

## Abstract

Netrin-1, a secreted protein recently characterized as an innovative cancer therapeutic target, is the anti-apoptotic ligand of Deleted in Colorectal Cancer (DCC) and UNC5H family member receptors, belonging to the dependence receptor family. As Netrin-1 is strongly expressed in several aggressive cancers, by inhibiting cell death induced by its receptors, interference of the binding with its receptors has been shown to actively induce apoptosis and tumour regression. To this line, a monoclonal neutralizing anti-Netrin-1 antibody has been developed and it is currently in phase I clinical trial. Recently, it has been shown that the transcription factor p53 positively regulates Netrin-1 gene expression.

Here we show that Netrin-1 up-regulation requires transcriptional activity of the second p53 transactivation domain. Moreover, we propose that Netrin-1 could be a new target gene of N-terminal p53 isoform  $\Delta 40p53$ , independently by full-length p53 activity. We demonstrate this hypothesis using stable cell lines, harbouring wild-type or null-p53, in which  $\Delta 40p53$  expression could be finely tuned. Our results indicate that  $\Delta 40p53$  is able to bind and activate Netrin-1 promoter, suggesting the formation of a  $\Delta 40p53$  homotetramer complex. In addition, employing CRISPR/Cas9 method, we show here that forcing immortalized human skeletal myoblasts cells to produce  $\Delta 40p53$  isoform, instead of full-length p53, leads to Netrin-1 and its receptor UNC5B up-regulation. As a consequence, cells increase Netrin-1 dependence to survival, allowing Netrin-1 inhibition to trigger apoptotic cell death. Finally, we clearly indicate a positive correlation between

Netrin-1 and  $\Delta 40p53$  gene expression in human melanoma and colorectal cancer biopsies.

In conclusion, we propose here a pro-tumour role of  $\Delta 40p53$  through direct activation of Netrin-1, independently to full-length p53 activity. Following dependence receptor notion, inhibition of Netrin-1 binding to its receptors, instead of targeting dominant-negative function, should be a promising therapeutic strategy in human tumours expressing high levels of  $\Delta 40p53$ .



## Introduction

Personalized cancer therapy treatment is based on direct targeting tumour biomarkers, usually proteins allowing cancer cells to survive. In the last years, several research works have highlighted the crucial role of the secreted protein Netrin-1 as innovative cancer biomarker and therapeutic target [1-3]. Initially discovered as axon guidance cue during neuronal development, Netrin-1 has also been involved in cell survival and tumorigenesis [4]. Indeed, Netrin-1 receptors Deleted in Colorectal Carcinoma (DCC) and UNC5 homologue (UNC5H, *i.e.* UNC5A-D) belong to the growing family of dependence receptors [5]. Such receptors share the peculiarity to be not only active upon ligand binding, inducing “positive” signals leading to differentiation and cell survival, but also to trigger apoptosis when unbound to the ligand [6]. For this reason, cell survival depends by the presence of dependence receptor ligands. Interestingly, a large part of the known dependence receptors are candidate tumour suppressors. The overall hypothesis is that the expression of such a receptor represents a protective mechanism that limits tumour development. This could be achieved by apoptosis induction of tumour cells that would grow or migrate beyond the regions of ligand availability [7]. Consequently, cell transformation toward malignant or metastatic phenotype could be associated with the constitutive inhibition of apoptosis induced by these receptors, *i.e.*, ligand ectopic expression. Along this line, Netrin-1 up-regulation has been described in several aggressive cancer [8-11]. Moreover, this expression increase has been associated with tumour progression [8,11,12]. Identification of Netrin-1 as tumour biomarker has allowed the development of an innovative personalized anti-cancer strategy. Indeed, interference of Netrin-1 and its receptor binding induces apoptosis and

favours tumour regression in several aggressive cancer models, expressing high Netrin-1 levels [1,8,10-12]. According to these findings, a humanized anti-Netrin-1 antibody, called NP137, has been developed [2] and is currently in a phase I clinical trial (<https://clinicaltrials.gov/ct2/show/NCT02977195>).

Despite the crucial importance of Netrin-1, little is known about Netrin-1 expression regulation. Recently, the transcription factor p53 has been involved in Netrin-1 gene expression regulation, upon chemotherapeutical treatment [1]. p53 is a well-known tumour suppressor gene, through its ability to regulate expression of different genes, involved in disparate cellular responses, including apoptosis, cell cycle arrest, senescence and metabolism [13]. p53 functions mainly depend by its ability to induce or repress transcription of target genes. However, transcription-independent activities of p53 has been described [14,15]. Transactivation activity of p53 requires two distinct transcriptional activation domains (TAD), comprising between residues 1-40 (TAD1) and 41-83 (TAD2) [16]. Experiments in mice carrying inactivating point mutations in one or both TADs have demonstrated a functional specialization of each TADs, that could regulate different target genes and effector pathways [17,18]. Interestingly, in the last few years, several p53 alternative transcripts were identified [19,20]. These protein isoforms are generated by different mechanisms, such as alternative splicing sites, promoters and translational initiation site [21,22]. Some of these p53 isoforms lack TAD1 ( $\Delta 40p53$ ) or both the transactivation domains ( $\Delta 133p53$ ) [20].  $\Delta 40p53$  isoform can be produced by alternative splicing, leading retention of intron 2 [23-25], or through the use of an alternative internal ribosomal entry sequences (IRES) at codon 40 [25]. Conversely,  $\Delta 133p53$  isoform is

generated from an internal promoter, located between intron 1 and exon 5, controlling the expression of a p53 mRNA starting from intron 4 [19,26].

Even if we have shown that full-length p53 (FLp53) directly binds and activates Netrin-1 promoter [1], the molecular mechanisms involved in this regulation are not well known. We show here that regulation of Netrin-1 and its main dependence receptor UNC5B requires p53 transactivation activity in tumour cells. Notably, Netrin-1 transcriptional activation requires only p53 TAD2, conversely to the majority of p53 target genes [17]. Consequently, we show that  $\Delta 40$ p53, and not  $\Delta 133$ p53, can positively regulate Netrin-1 and UNC5B gene expression, in a FLp53-independent manner. Moreover, we demonstrate that expression of  $\Delta 40$ p53 and Netrin-1 are correlated in human tumours. Finally, we show that treatment of tumour cells expressing  $\Delta 40$ p53 with neutralizing anti-Netrin-1 antibody could be an innovative anti-tumour therapeutic strategy.

## **Materials and Methods**

### **Cell Lines**

Human lung A549 cancer cell line, obtained from ATCC, were cultured in Dulbecco's modified Eagle's (DMEM) medium (Life Technologies, Carlsbad, CA, USA) containing 10% foetal bovine serum (Life Technologies) and 50µg/mL Gentamycin (Life Technologies). Human lung H1299 cancer cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 + Glutamax medium (Life Technologies) containing 10% foetal bovine serum and 50µg/mL Gentamycin (Life Technologies). Human skeletal myoblasts LHCN-M2 cell line was cultured in medium (four parts of DMEM medium to one part medium 199), supplemented with 20% foetal bovine serum (Life Technologies), 30 mM HEPES and 5 ng/mL HGF (R&D systems, Minneapolis, MN, USA), 1 µM dexamethasone (Sigma-Aldrich, Saint Quentin-Fallavier, France), 1.4 mg/L zinc sulphate (ZnSO<sub>4</sub>, Sigma-Aldrich) and 0.03 mg/L vitamin B12 (Sigma-Aldrich). A549 and H1299 cell lines were obtained from ATCC (Manassas, VA, USA). hTERT and CDK4-immortalized LHCN-M2 cells were a kind gift of Drs Chun-Hong Zhu and Woodring E. Wright [27]. All cell lines were regularly tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

### **Reagents and transfection procedures**

Doxycycline hyclate and doxorubicin hydrochloride were obtained from Sigma-Aldrich. 21nt-siRNA were designed using Designer of Small Interfering RNA (DSIR) tool [28], available at <http://biodev.extra.cea.fr/DSIR/DSIR.html> site. siRNA oligonucleotides

were synthesized by Sigma-Aldrich. siRNA transfection of LHCN-M2 was performed using lipofectamine RNAiMAX reagent (Life Technologies). For plasmid transfection to generate stable cell lines, A549 and H1299 cell lines were transfected using Jet Prime reagent (PolyPlus-Transfection, Illkirch, France). All transfections were performed following manufacturer instructions.

### **Generation of stable cell lines**

For the generation of inducible stable cell lines, HA-tagged human FLp53,  $\Delta$ 40p53 and  $\Delta$ 133p53 coding sequences were cloned at SfiI sites in the pITR plasmid, a sleeping beauty-based vector allowing the doxycycline inducible expression of p53 isoforms, 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 [29]. Alternatively, p53 isoforms sequences were cloned in pITR2 plasmid, a modified version of pITR vector, containing Zeocin resistance gene and mCherry fluorescent protein. A549 and H1299 cell lines were co-transfected with p53 isoforms-pITR/pITR2 plasmid and a sleeping beauty transposase expression vector (SB100X). pITR vector was a generous gift of Prof. Rolf Marschalek (Goethe-University of Frankfurt, Germany). Stable cell clones were selected for 3 days in puromycin-containing medium. Alternatively, stable cell lines were sorted by flow cytometry to selected GFP or mCherry positive cells. All the stable inducible cell lines were further tested for p53 isoforms expression in response to doxycycline. Plasmids containing cDNA sequence of FL53,  $\Delta$ 40p53 and  $\Delta$ 133p53 cDNA were a kind gift of Dr Jean-Christophe Bourdon (University of Dundee, Scotland, UK).

For generation of stable LHCN-M2 cell line, knocked-out for FLp53 or total p53, small guide RNAs (sgRNA) were designed in order to selectively target ATG codon in position 1 (to knock-out only FLp53) or in position 40 (to knock-out total p53). 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 [30]. 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. For generation of A549 stable cell line, knocked-out for endogenous FLp53, sgRNA were designed using the GPP (Genetic Perturbation Platform, Broad Institute) sgRNA Designer tool (CRISPRa/i), available at <https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-crisprai> site. sgRNAs, designed close to p53 promoter, were annealed as described above and inserted in pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro vector [31], allowing expression of defective Cas9 enzyme (dCas9) fused to the Krüppel-associated box (KRAB) transcriptional repressor (dCas9-KRAB). plentiCRISPR v2 was a gift from Feng Zhang (Addgene, Watertown, MA, USA, plasmid #52961; <http://n2t.net/addgene:52961>; RRID:Addgene\_52961), while pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro was a gift from Charles Gersbach (Addgene, plasmid #71236; <http://n2t.net/addgene:71236>; RRID:Addgene\_71236).

### **Quantitative RT-PCR**

Total RNAs were extracted using NucleoSpin® RNA Plus Kit (Macherey Nagel, Düren, Germany) according to manufacturer's protocol. RT-PCR reactions were

performed with PrimeScript RT Reagent Kit (Takara Bio Europe, Saint-Germain-en-Laye, France). 500 ng 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, Indianapolis, IN, USA), using the Premix Ex Taq probe qPCR Kit (Takara Bio Europe), according to manufacturer instructions. Expression of target genes was normalized to TATA binding protein (TBP) and beta-glucuronidase (GUSB) genes, used as housekeeping genes. The amount of target transcripts, normalized to the housekeeping gene, was calculated using the comparative  $C_T$  method. A validation experiment was performed, in order to demonstrate that efficiencies of target and housekeeping genes were approximately equal. Sequences of the primers are available upon request.

### **Western Blotting Analysis**

For immunoblotting analysis, cells were lysed by sonication in SDS buffer (10mM Tris-HCl pH 7.4, 10% glycerol, 5% SDS, 1% TX-100, 100mM DTT) in the presence of protease inhibitor cocktail (Roche Applied Science). Protein extracts (20-50µg per lane) were loaded onto 4-15% SDS-polyacrylamide gels (Biorad, Hercules, CA, USA) and blotted onto nitrocellulose sheets using Trans-Blot Turbo Transfer System (Biorad). Filters were blocked with 10% non-fat dried milk in PBS/0.1% Tween 20 (PBS-T) for 2 hours and then incubated over-night at 4°C with the following antibody: rabbit monoclonal antibody  $\alpha$ -Netrin-1 (1:1000 dilution, #ab126729, Abcam, Cambridge, UK,); rabbit polyclonal antibody  $\alpha$ -p21 (1:2000, clone C-19, #sc-397, Santa Cruz Biotechnology, Dallas, TX, USA,); mouse monoclonal antibody  $\alpha$ -p53 (1:1000, clone DO-1, #sc-126, Santa Cruz

Biotechnology); mouse monoclonal antibody  $\alpha$ -GAPDH (1:1000, clone 0411, #sc-47724, Santa Cruz Biotechnology); rabbit polyclonal antibody  $\alpha$ -HA (1:5000, #H6908, Sigma-Aldrich); rabbit monoclonal antibody  $\alpha$ -BAX (1:1000, clone D2E11, #5023, Cell Signaling, Danvers, MA, USA); rabbit monoclonal antibody  $\alpha$ -Ku80 (1:1000, clone C48E7, #2180, Cell Signaling); mouse monoclonal antibody  $\alpha$ - $\beta$ -actin, HRP-conjugated (1:10000, clone BA3R, #MA5-15739-HRP, Life Technologies); mouse monoclonal antibody  $\alpha$ -p53 (1:1000, clone DO-11, #MCA1704, Biorad); rabbit monoclonal antibody  $\alpha$ -UNC5B (1:1000, clone D9M7Z, # 13851, Cell Signaling). 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 (Life Technologies). Membranes were imaged on the ChemiDoc Touch Imaging System (Biorad).

## **Reporter Assay**

Stable A549 cells inducible for FLp53 or  $\Delta$ 40p53 were plated in 12-well plates and transfected with the different firefly luciferase reporters containing wild-type or p53-mutated Netrin-1 promoter constructs, described before [1,9,32]. 24 hours after transfection, cells were treated with 2 $\mu$ g/mL Doxycycline and further incubated for 24 hours. All transfections were performed in triplicate and the Dual-Luciferase Reporter Assay system (Promega, Charbonnières, France) was carried out 48h after transfection according to the manufacturer's protocol, using the Infinite F500 apparatus (Tecan). As an internal control of transfection efficiency, the Renilla luciferase encoding plasmid (pRL-



CMV, Promega) was co-transfected and for each sample firefly luciferase activity was normalized to the Renilla luciferase activity.

### **Cell Death Assays**

For DNA fragmentation analysis (SubG1), LHCN-M2 cell lines were plated onto 6-well-plates at a density of  $1.5 \times 10^5$  cells per well. The following day, cells were transfected with siRNA, as described above, and incubated in non-serum medium for 48 hours. Then cells were collected, fixed in 70% ethanol and incubated at  $-20^{\circ}\text{C}$  for at least one night. Further, cells were washed twice with PBS and incubated for 5 min at  $37^{\circ}\text{C}$  in PBS containing 0.1% TX-100 and  $2\mu\text{g}/\text{mL}$  4',6-diamidino-2-phenylindole (DAPI). DNA content was evaluated by NucleoCounter NC-3000 system (ChemoMetec A/S, Allerød, Denmark).

Caspase-3 activity assay was performed as described previously [29]. Briefly, LHCN-M2 cells were seeded and treated as for DNA fragmentation assay. Cells were then collected and caspase-3 activity assay was performed using the Caspase-3 Fluorometric Assay Kit (BioVision, Milpitas, CA, USA), 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, Männedorf, Switzerland).

### **Patients and tumour samples**

For melanoma cohort, 28 tumour samples were collected as previously described [33], from patients underwent surgery for skin melanocytic lesions between 2007 and 2011. The research protocol was approved by the Ethics committee of Saint Louis

Hospital (Paris, France). 21 primary colorectal cancer tissue samples were obtained from the Biological Resource Centre (CRB, Centre de Resource Biologique) of Léon Bérard Centre (protocol number: BB-0033-00050) (Lyon, France), as previously described [34]. Research protocol was approved by CRB medical and scientific committee. According to the French Bioethics law, all the patients signed consent informing them the research use of the samples.

### **Statistical analysis**

The data reported are the mean  $\pm$  S.D. of at least three independent determinations, each performed in triplicate. Statistical analysis was performed by the Two-way ANOVA or linear regression tests, using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA).

## Results

### **Netrin-1 regulation by p53 requires transcriptional activity of TA2 domain**

p53 can regulate gene expression independently by its transcriptional activity [14,15]. Moreover, p53 N-terminal transactivation domains (TAD) are able to regulate different gene sets [17]. Indeed, site-specific L22Q and W23S mutations in TAD1 significantly inhibit transcriptional activation by p53 protein [35], while W53Q and F54S mutations inactivate TAD2 (Fig. 1a) [16]. To investigate how Netrin-1 is regulated by p53, we use sleeping beauty-based vectors to generate stable lung cancer H1299 cell lines, that are p53-null cells, in which wild-type or transcriptional inactivated p53 mutants are expressed upon treatment with doxycycline. Interestingly, mutants p53 proteins, in particular TAD1 mutants, are more stable than wild-type p53, a particularity already described before [17,35,36]. As previously showed [1], over-expression of wild-type p53 strongly induced Netrin-1, as well as a typical p53 target gene, such as p21 (Fig. 1b). However, while inactivation of p53 TAD1 did not affect Netrin-1 protein expression, over-expression of TAD2 mutant p53, alone or in combination with TAD1 mutant protein, was not sufficient to induce Netrin-1 (Fig. 1b). We confirmed by quantitative PCR the requirement of TAD2 p53 to trigger Netrin-1 transcript (Fig. 1c). These results suggest that Netrin-1 up-regulation requires p53 transcriptional activity.

### **$\Delta 40p53$ , but not $\Delta 133p53$ , regulates Netrin-1 and UNC5B gene expression**

Some of the p53 alternative transcripts, generated by 5' splicing, IRES or alternative promoter, lack TAD1 ( $\Delta 40p53$ ) or both TADs ( $\Delta 133p53$ ) (Fig. 2a). These particularities render these p53 isoforms similar to transcriptional mutants we generated.

Then, we wondered whether Netrin-1 gene expression was regulated by N-terminal p53 isoforms. To this purpose, we generated stable lung cancer A549 cell lines, harbouring wild-type p53, inducible for  $\Delta 40p53$  or  $\Delta 133p53$  isoforms and we analysed gene expression by quantitative PCR. Upon treatment with doxycycline,  $\Delta 40p53$  over-expression strongly activated Netrin-1 gene expression, while we noticed no effect upon  $\Delta 133p53$  induction (Fig. 2b, left panel), confirming results obtained with p53 transcriptional mutants. Interestingly,  $\Delta 40p53$ , but not  $\Delta 133p53$ , was also able to up-regulate the main Netrin-1 receptor, UNC5B (Fig. 2b, middle panel). Moreover, despite  $\Delta 40p53$  has been described to negatively modulate p21 [37,38], we observed a positive regulation of p21 by  $\Delta 40p53$ , at least in our inducible cell lines (Fig. 2b, right panel). We confirmed by western blot the positive regulation of Netrin-1 and p21 by  $\Delta 40p53$  induction at protein levels (Fig. 2c). Also, we noticed no effect of  $\Delta 40p53$  over-expression on BAX protein (Fig. 2c), a typical p53 target gene involved in p53 pro-apoptotic function, conversely to p21, involved in p53 cell cycle arrest.

To confirm that Netrin-1 induction by  $\Delta 40p53$  depended by its transcriptional activity, we mutated  $\Delta 40p53$  TAD, corresponding to FLp53 TAD2 (Fig. 2a), in the amino acid residues W14 and F15 ( $\Delta 40p53^{14,15}$ ), equivalent to position 53 and 54 in FLp53. Then, we generated stable A549 cells inducible for wild-type  $\Delta 40p53$  and  $\Delta 40p53^{14,15}$  upon treatment with doxycycline and we assessed protein and transcript levels by western blot and quantitative PCR. Results showed that deregulation of  $\Delta 40p53$  transcriptional activity strongly inhibited protein increase of Netrin-1 and p21 observed upon induction of  $\Delta 40p53$  proteins (Fig. 2d). Moreover,  $\Delta 40p53^{14,15}$  over-expression weakly increased Netrin-1,

UNC5B and p21 gene expression, compared to wild-type  $\Delta 40p53$  (Supplementary Figure 1a).

As A549 cells we used to induce N-terminal p53 isoforms harbour wild-type p53, we wondered whether their expression could affect gene expression upon Doxorubicin treatment and consequent endogenous FLp53 accumulation and activation. To this purpose, we treated  $\Delta 40p53$ - and  $\Delta 133p53$ -A549 stable cells with both doxycycline and Doxorubicin and we analysed gene expression by quantitative PCR. In  $\Delta 40p53$ -A549 cells, Netrin-1, UNC5B and p21 gene expression was strongly up-regulated upon treatment with doxycycline and Doxorubicin separately (Fig. 2e), confirming that  $\Delta 40p53$  is able to positively regulate these genes, as well as that endogenous FLp53, induced by Doxorubicin, triggers Netrin-1 and UNC5B gene expression [1]. Interestingly, Netrin-1 and UNC5B up-regulation triggered by Doxorubicin further increased upon induction of  $\Delta 40p53$ , while in  $\Delta 133p53$ -A549 cells doxycycline treatment strongly inhibited Netrin-1 and UNC5B induction by Doxorubicin (Fig. 2e), confirming a dominant-negative role of  $\Delta 133p53$  against FLp53 [19,39]. Intriguingly, p21 gene expression following Doxorubicin treatment was suppressed by doxycycline in both  $\Delta 40p53$ - and  $\Delta 133p53$ -A549 cells (Fig. 2e), suggesting that, at least for p21,  $\Delta 40p53$  could also act as a dominant-negative effector of FLp53.

### **FLp53 is not necessary for Netrin-1 regulation by $\Delta 40p53$**

Even if increasing evidences suggest that  $\Delta 40p53$  could regulate gene expression independently by FLp53 [40-42], this isoform could also function as a FLp53 regulator [25]. Indeed,  $\Delta 40p53$  can oligomerize with FLp53, regulating its activities and functions

[25]. To assess the involvement of FLp53 on  $\Delta$ 40p53-dependent regulation of Netrin-1, we generated stable p53-null H1299 cells, inducible for  $\Delta$ 40p53, FLp53 or both proteins. Gene expression analysis upon doxycycline treatment revealed a robust induction of Netrin-1 gene expression following over-expression of both p53 proteins (Fig. 3a). Moreover, co-expression of FLp53 and  $\Delta$ 40p53 did not significantly change Netrin-1 expression, compared to separate inductions. We confirmed by western blot strong Netrin-1 up-regulation, as well as p21 increase upon p53 proteins induction (Fig. 3b).

To confirm that  $\Delta$ 40p53 could regulate Netrin-1 *per se*, regardless to FLp53 expression, we silenced endogenous FLp53 expression in A549 cells inducible for  $\Delta$ 40p53, using a specific siRNA targeting exon 2 of p53 gene, thus avoid degradation of induced  $\Delta$ 40p53 mRNA. To allow accumulation of endogenous FLp53, cells were treated with Doxorubicin, in addition to doxycycline to induce  $\Delta$ 40p53 expression. FLp53 silencing was then verified by quantitative PCR (Supplementary Figure 1b). Western blot and quantitative PCR analysis showed a strong accumulation of Netrin-1, UNC5B and p21 upon induction of  $\Delta$ 40p53 or Doxorubicin treatment in cells transfected with control siRNA (Fig. 3c, d and Supplementary Figure 1b). However, FLp53 silencing completely abolished target gene increases observed in Doxorubicin-treated cells. By contrast,  $\Delta$ 40p53 was able to trigger Netrin-1, UNC5B and p21 gene expression also in cells transfected with FLp53 siRNA (Fig. 3c, d and Supplementary Figure 1b).

Finally, to avoid siRNA transfection and confirm the  $\Delta$ 40p53 autonomy to FLp53 to induce Netrin-1, we stably knocked-out endogenous FLp53 in A549 cells inducible for  $\Delta$ 40p53, using CRISPR-dCas9-KRAB, a modified CRISPR/Cas9-based system [31]. We designed small guides RNA (sgRNAs), targeting p53 promoter, able to direct the binding

of a nuclease defective Cas9 (dCas9) enzyme, fused to KRAB (Krüppel-associated box) domain of Kox1 [31,43]. KRAB domain allowed transcription repression of p53 by mediating local epigenetic reprogramming of histone modifications [44,45]. FLp53 down-regulation was verified by western blot (Fig. 3c) in stable A549 cells treated with Doxorubicin. sgp53A completely knocked-out basal and drugs-induced FLp53, while sgp53B showed a robust repression of FLp53 expression. Accordingly, Netrin-1 up-regulation following Doxorubicin treatment was completely inhibited in FLp53 knock-out cells at protein and transcriptional levels (Fig. 3c and Supplementary Figure 2a), as well as its receptor UNC5B (Supplementary Figure 2b). However,  $\Delta 40p53$  induction by doxycycline was still sufficient to trigger Netrin-1 and UNC5B gene expression, also in the absence of FLp53. Collectively, these results demonstrate that Netrin-1 induction by  $\Delta 40p53$  does not require FLp53.

### **$\Delta 40p53$ directly binds and activates Netrin-1 promoter**

We then assessed if  $\Delta 40p53$  was able to regulate Netrin-1 promoter activity. Indeed, we have previously cloned two different Netrin-1 promoters [9,32], and we have confirmed the presence of a p53 binding site in the internal Netrin-1 promoter [1]. As shown in Figure 4, FLp53 or  $\Delta 40p53$  induction upon doxycycline treatment triggered transcription of firefly luciferase reporter gene placed under the control of the internal Netrin-1 promoter or p21 promoter. However, site-direct mutagenesis of p53 binding site in Netrin-1 promoter completely abolished promoter transactivation upon induction of both FLp53 and  $\Delta 40p53$  (Fig. 4).

## **Endogenous $\Delta 40p53$ regulates Netrin-1 expression**

Until now, we have shown that over-expressed  $\Delta 40p53$  induces Netrin-1 and its receptor UNC5B gene expression. To study the role of endogenous-produced  $\Delta 40p53$  on Netrin-1 regulation, we used immortalized human skeletal myoblasts LHCN-M2 cell line, harbouring wild-type p53. We designed two different sgRNAs, targeting first “canonical” ATG in p53 exon 2 (sgp53#10) and ATG codon 40 in p53 exon 4 (sgp53#400), in order to inactivate only FLp53 or FL/ $\Delta 40p53$  isoforms, respectively (Fig. 5a). Then, using CRISPR-Cas9 technique, we generated stable LHCN-M2 cell lines. Western blot analysis revealed a complete knock-out of p53 isoforms in stable cells generated with sgp53#400, while in cells set-up with sgp53#10 only FLp53 was deleted (Fig. 5b). Moreover, in these cells a band corresponding to  $\Delta 40p53$  appeared, demonstrating that this CRISPR-Cas9 strategy allowed us to force LHCN-M2 cells to produce endogenous  $\Delta 40p53$  instead of FLp53. Interestingly, Netrin-1 and UNC5B proteins were strongly expressed in LHCN-M2-sgp53#10, expressing  $\Delta 40p53$  in place of FLp53, compared to control or sgp53#400 cells (Fig. 5b). Moreover, as described in Figure 5c, quantitative PCR analysis confirmed Netrin-1 increase in LHCN-M2 cells expressing endogenous  $\Delta 40p53$ . Intriguingly, gene expression of p21 and GADD45 (p53 target genes involved in cell cycle arrest) strongly decreased in both FLp53 knock-out cells, compared to parental or control LHCN-M2 cells, while only complete p53 knock-out drove BAX (involved in apoptotic function of p53) gene expression down (Fig. 5c and Supplementary Figure 3).

## **$\Delta 40p53$ expression amplifies Netrin-1 dependence and sensitizes cells to Netrin-1 interference-induced apoptosis**



Since over-expression or forced expression of  $\Delta 40p53$  triggered both Netrin-1 and its receptor UNC5B gene expression, we could hypothesize that in these cells dependence to Netrin-1 expression should be amplified. To confirm this hypothesis, we transfected stable LHCN-M2 cells described before with siRNA specific for Netrin-1, in order to silence Netrin-1, as described before [1]. As shown in Figure 6a, Netrin-1 silencing induced an increase of DNA fragmentation only in sgp53#10 cells, expressing endogenous  $\Delta 40p53$  and consequently Netrin-1 and UNC5B. Moreover, in these cells Netrin-1 siRNA transfection induced a robust caspase-3 activation, compared to parental or control LHCN-M2 cells (Fig. 6b). Also, complete p53 knock-out desensitized sgp53#400 cells to Netrin-1 silencing (Fig. 6a, b).

### **Netrin-1 and $\Delta 40p53$ gene expression correlates in human tumour biopsies**

Conversely to FLp53, whose activity in human cancers is mainly regulated at post-translational level,  $\Delta 40p53$  gene expression could variate and contribute to neoplastic phenotype [37,46-48]. As Netrin-1 is often deregulated in cancer progression, we wondered whether  $\Delta 40p53$  could positively regulate Netrin-1 in a panel of human tumours. To this purpose, we analysed Netrin-1 and  $\Delta 40p53$  gene expression in melanoma and colorectal cancer biopsies. As shown in Figure 7a, in both the cohorts we analysed, we found a positive and significant correlation between Netrin-1 and  $\Delta 40p53$ . Moreover, Netrin-1 resulted more expressed in human tumour biopsies presenting high levels of  $\Delta 40p53$  (Fig. 7b). These results suggest that in human tumour biopsies Netrin-1 could be positively regulated by  $\Delta 40p53$ .



## Discussion

Netrin-1 is a protein frequently up-regulated in aggressive human cancers [8-11]. Netrin-1 pro-tumour function is resulting mainly from its ability to bind dependence receptors DCC and UNC5H, inhibiting their pro-apoptotic activity [5,49-52]. Within this context, several efforts focused on inhibition of Netrin-1 and its receptors binding to induce cancer cell death and tumour regression [2,8,10-12,53]. These attempts allowed the development of a Netrin-1 neutralizing antibody, currently in Phase I clinical trial [2]. However, Netrin-1 inhibition strategy could be only applied in the fraction of tumours expressing high levels of Netrin-1. Therefore, the knowledge of which cancer cells have selected this strategy to survive and what are the mechanisms involved in Netrin-1 up-regulation in tumour cells could be important in developing personalized therapeutic treatments. Indeed, we have recently demonstrated that the transcription factor p53 regulates Netrin-1 [1]. This knowledge could be important to increase the fraction of cancer patients who would be eligible to a Netrin-1 interference-based treatment during early clinical evaluation.

Here we propose that  $\Delta 40p53$ , a N-terminal truncated isoform of p53, could transcriptionally regulate Netrin-1 and its receptor UNC5B gene expression. Interesting, this regulation seems to be independent by the presence of FLp53. Indeed, early studies pointed out  $\Delta 40p53$  as a dominant-negative regulator of FLp53 [20]. For instance,  $\Delta 40p53$  over-expression down-regulates FLp53-induced target genes, counteracting FLp53-dependent growth suppression [24,25]. However, more recently  $\Delta 40p53$  has been described as a positive regulator of FLp53 functions [38]. Indeed, in melanoma cells,  $\Delta 40p53$  induces apoptosis, but only in cells expressing wild-type FLp53 [37]. These

contradictory results have been explained by dose-dependent effect of hetero-oligomerization of p53 isoforms, dependent on the relative expression levels [54]. For instance, it has been demonstrated that low  $\Delta 40p53$  expression enhances FLp53 transactivation activity, while increasing levels suppress anti-proliferative effect of FLp53 [38]. As Netrin-1 has been already described as a FLp53 target gene [1], its regulation by  $\Delta 40p53$  expression, finely tuned by inducible sleeping beauty system we used, could be barely due to p53 stabilization and activation by hetero-oligomers  $\Delta 40p53/FLp53$ . Indeed, hetero-tetramer complex containing  $\Delta 40p53$  and FLp53 has been described more stable than FLp53 homo-tetramers [55], probably by protecting p53 complex to MDM2-mediated degradation [25,41]. However, our results, showing Netrin-1 up-regulation by  $\Delta 40p53$  in H1299 p53-null cells or upon FLp53 knock-out in A549 p53-wild-type cells, suggest the formation of a  $\Delta 40p53$ -only homo-tetramer, binding and activating Netrin-1 promoter. On the other hand, binding of  $\Delta 40p53$  to p53 responsive elements located in MDM2, GADD45 and BAX genes, leading to FLp53-independent transactivation, has been already reported [41]. Interesting, our data also show that CRISPR/Cas9 technique, using specific sgRNAs, allow us to generate stable cells expressing  $\Delta 40p53$  instead of FLp53. In this way, we were able to confirm up-regulation of Netrin-1 and UNC5B also by endogenous-expressed  $\Delta 40p53$ . This information, together with gene expression analysis on inducible stable cell lines, suggest that  $\Delta 40p53$  can establish homo-tetramers, transactivating Netrin-1 promoter, in both high and low  $\Delta 40p53$  expression levels, at least in the absence of FLp53.

p53 isoforms are differentially expressed in several human cancers [21,56]. For example,  $\Delta 40p53$  results up-regulated in tumour breast tissue, compared to normal breast, and associated with aggressive triple-negative breast cancer [48], as well as in mucinous ovarian cancer [46]. Moreover,  $\Delta 40p53$  isoform is well-expressed in melanoma

cell lines and biopsies, while undetectable in melanocytes and normal tissue [47]. However, high expression and cancer aggressiveness correlated to  $\Delta 40p53$  has been associated to dominant-negative function of this isoform on FLp53 activity. Interesting, we show here a positive correlation between  $\Delta 40p53$  and Netrin-1 gene expression in melanoma and colorectal cancer cohorts. Moreover, Netrin-1 and its dependence receptors have been involved in melanoma and colorectal cancer progression [9,33,52,57]. Then, our data could suggest for the first time a FLp53-independent pro-tumour role of  $\Delta 40p53$ , since its ability to transactivate an anti-apoptotic protein such as Netrin-1.

Besides high expression in human cancers,  $\Delta 40p53$  has been recently involved in controlling pluripotency in embryonic stem cells (ESCs). Indeed, this isoform is highly expressed in ESCs, regulating progression from pluripotency and differentiation in these cells [58]. Moreover,  $\Delta 40p53$  inhibits loss of pluripotency associated with FLp53 activation, and promotes ESCs survival [58]. Again, it has been proposed that  $\Delta 40p53$  role in ESCs is merely to suppress FLp53 activity, allowing pluripotency transcription factors to accumulate [58]. Interesting, Netrin-1 has also been involved in pluripotency control in ESCs. A recent paper shows that Netrin-1 expression enhances mouse ESCs self-renewal, by blocking UNC5B-induced cell death [59]. We could then suggest that  $\Delta 40p53$  promotes ESCs survival by regulating Netrin-1 expression, allowing regulation of pluripotency.

Regulation of Netrin-1 and UNC5B receptor by  $\Delta 40p53$  isoform could have significant therapeutic consequence. Indeed, the fact that both the ligand and its dependence receptor are up-regulated in human cancer co-expressing  $\Delta 40p53$  could enhance dependence of tumour cell survival to Netrin-1 expression. Specifically, inhibition

of interference of Netrin-1 in these cancer cells should lead to dependence receptor-induced apoptosis and then tumour regression. Our results demonstrate that silencing of Netrin-1 expression by RNA interference induces cell death only in cells that are forced to produce  $\Delta 40p53$  and consequently express high levels of Netrin-1 and UNC5B. From a therapeutic point of view, this information suggests that interference of the binding between Netrin-1 and UNC5B with a more therapeutic relevant treatment, such as anti-Netrin-1 neutralizing antibody we developed in our laboratory, should be more efficient in human tumours expressing high levels of  $\Delta 40p53$ .

In conclusion, we show here for the first time that  $\Delta 40p53$  isoform could positively regulate an anti-apoptotic protein, such as Netrin-1, in a FLp53-independent way. In addition, this regulation, achieved by both over-expressed or endogenous  $\Delta 40p53$ , could explain increased transcript levels of this protein, observed in several human cancers. Indeed, each mutation changing the ratio between FLp53 and  $\Delta 40p53$  generation could confer a selective advantage to cancer cells. Of course,  $\Delta 40p53$  could inhibit tumour suppressor activity of FLp53, acting as a dominant-negative protein, however it could also actively transactivate pro-tumour factor, such as Netrin-1, as our results seem to suggest. In this situation, a promising therapeutic strategy could be to inhibit pro-tumour factor activity, as we propose for Netrin-1, instead of targeting dominant-negative function of  $\Delta 40p53$ .

## **Acknowledgements**

We wish to thank Dr Jean-Christophe Bourdon for p53-related materials. This work was supported by institutional grants from CNRS, University of Lyon, Centre Léon Bérard and from the Ligue Contre le Cancer, INCA, ANR, ERC and Fondation Bettencourt. Yan Sun and Hong Wang have been supported by a fellowship from China Scholarship Council, Ambroise Manceau from Ligue Contre le Cancer, Valeria Basso from Nuovo-Soldati Cancer Research Foundation.

## **Conflict of Interest**

PM declares to have a conflict of interest in the study as a founder and shareholder of Netris Pharma. The remaining authors declare that they have no conflict of interest.

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## Figure Legends

### Figure 1. Netrin-1 regulation by p53 requires TA2 domain.

**(a):** Schematic representation of p53 domain structure. p53 domain structure can be divided in three components: an N-terminal domain, including two distinct transactivation domains (TAD1 and TAD2) and a proline-rich domain (PXXP); a core domain with the DNA binding domain, accounting for binding to responsive elements in a promoter; a C-terminal domain, containing an oligomerization domain, allowing p53 tetramerization, and a basic domain required for protein stability. In red are indicated mutant p53 proteins with corresponding mutations allowing inactivation of TAD1 or TAD2. **(b,c):** TAD2 mutant p53 proteins are not able to trigger Netrin-1. Stable H1299 cell lines, inducible for wild-type or mutant p53 proteins, were treated with Doxycycline for 24 hours and expression of Netrin-1, p21 and p53 was analyzed by western blot (b) or quantitative PCR (c). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was used to normalize western blot, while quantitative PCR was normalized using TATA binding protein (TBP) and  $\beta$ -glucuronidase (GUSB) as housekeeping genes. Data are presented as mean  $\pm$  SD (n=3). \*, p < 0.05; \*\*, p < 0.01. NT, not treated; Dox, doxycycline; NTN1, Netrin-1.

### Figure 2. $\Delta$ 40p53, but not $\Delta$ 133p53, regulates Netrin-1 and UNC5B expression.

**(a):** Schematic representation of p53 isoform domain structure.  $\Delta$ 40p53 isoform lacks TAD1 (in yellow), while  $\Delta$ 133p53 results deleted of the entire N-terminal domain (including TAD1, TAD2 and PXXP domains) and a small part of DNA binding domain (in dark blue).

**(b,c):** Over-expression of  $\Delta 40p53$  is sufficient to induce Netrin-1 gene expression. Stable A549 cell lines, inducible for empty vector (pITR),  $\Delta 40p53$ -HA and  $\Delta 133p53$ -HA, were treated with Doxycycline for 24 hours. Netrin-1, UNC5B and p21 gene expression was analyzed by quantitative PCR (b), while protein expression of Netrin-1, p21 and BAX was evaluated by western blot (c). Expression of HA-tagged p53 isoforms was also assessed by western blot. **(d):**  $\Delta 40p53$ -dependent Netrin-1 induction requires  $\Delta 40p53$  transcriptional activity. Stable A549 cell lines, inducible for wild-type or TAD-mutated ( $\Delta 40p53^{14,15}$ )  $\Delta 40p53$  protein, were treated with Doxycycline. Netrin-1, p21 and p53 protein expression was evaluated by western blot. Ku80 protein was used to normalize protein expression **(e):**  $\Delta 40p53$  expression, but not  $\Delta 133p53$ , increases Doxorubicin-induced Netrin-1 up-regulation, while decreases p21 gene expression upon Doxorubicin treatment. Stable A549 cells inducible for  $\Delta 40p53$  or  $\Delta 133p53$  were treated with Doxycycline and/or Doxorubicin for 24 hours. Expression levels of Netrin-1 (NTN1), UNC5B and p21 were evaluated by quantitative PCR. Expression of TBP and GUSB was used as housekeeping genes. Data are presented as mean  $\pm$  SD (n=3). \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001; \*\*\*\*, p < 0.0001. NT, not treated; Dox, doxycycline; NTN1, Netrin-1; HA, influenza hemagglutinin.

**Figure 3. Full-length p53 is not necessary for Netrin-1 regulation by  $\Delta 40p53$ .**

**(a,b):** Over-expression of full-length p53 or  $\Delta 40p53$  in p53-null cells is sufficient to induce Netrin-1 expression. Stable p53-null H1299 cell lines, inducible for full-length p53 (p53FL) or  $\Delta 40p53$  proteins, alone or in combination (p53FL+ $\Delta 40p53$ ), were treated with

Doxycycline for 24 hours. Netrin-1 (NTN1) gene expression was assessed by quantitative PCR (a). Protein levels of Netrin-1 and p21, as well as p53FL and  $\Delta$ 40p53, were evaluated by western blot (b). **(c,d)**: Silencing endogenous p53 expression does not affect  $\Delta$ 40p53-dependent Netrin-1 up-regulation. Stable A549 cell lines, inducible for  $\Delta$ 40p53, were transfected with a scramble siRNA (siCTRL) or with a specific siRNA targeting exon 2 of p53 gene (sip53). 24 hours after transfection, cells were treated with Doxorubicin or doxycycline for further 24 hours. Western blot assay (c) allowed analysis of Netrin-1 protein levels, while Netrin-1 (NTN1) transcript levels were measured by quantitative PCR (d). **(e)**:  $\Delta$ 40p53-induced Netrin-1 gene expression is not altered by knocking-down endogenous p53. A549 cells were infected using CRISPR/Cas9-modified pLV-hU6-sgRNA-hUbc-dCas9-KRAB-T2a-Puro plasmid, and stable cell lines were generated using firefly luciferase-control (sgLUC) or p53-specific (sgp53A and sgp53B) small guide RNAs. Endogenous p53 knockout cell lines were then transfected with empty (pITR) or  $\Delta$ 40p53 sleeping-beauty plasmids. Stable A549 cell lines knockout for p53 and inducible for  $\Delta$ 40p53 were treated with Doxorubicin and Doxycycline for 24 hours. Efficiency of endogenous p53 knockout was evaluated by western blot, using p53-DO1 antibody, while anti-HA antibody allowed verification of  $\Delta$ 40p53 expression. In addition, Netrin-1 and p21 protein levels were assessed by western blot. Data are representative of at least three replicates. Quantitative PCR results are presented as mean  $\pm$  SD (n=4). ns, non-significant; \*\*, p < 0.01; \*\*\*, p < 0.001; \*\*\*\*, p < 0.0001. NT, not treated; Dox, doxycycline; DoxoR, Doxorubicin; NTN1, Netrin-1; HA, influenza hemagglutinin; sg, small guide; FLp53, full-length p53.



**Figure 4.  $\Delta 40p53$  directly binds and activates Netrin-1 promoter.**

$\Delta 40p53$  activates Netrin-1 promoter, binding to canonical p53 responsive elements. Stable A549 cells inducible for  $\Delta 40p53$  or full-length p53 (FLp53) were transfected with wild-type (NetP) or mutated for p53 binding site (NetP mut) promoters, as well as p21 promoter (p21P). 48 hours after transfection, promoter activities were assessed by luciferase assay. Values represent mean  $\pm$  SD (n=3) \*\*\*, p < 0.001; \*\*\*\*, p < 0.0001; CTRL, control; Dox, doxycycline; FLp53, full-length p53; NetP, Netrin-1 promoter; p21P, p21 promoter.

**Figure 5. Endogenous  $\Delta 40p53$  regulates Netrin-1 expression.**

**(a):** Schematic representation of 5' sequence of p53 gene. ATG codons for full-length p53 (ATG1) and  $\Delta 40p53$  (ATG40) are indicated, as well as two small guide RNAs (sgRNAs), designed near ATG1 (guide#10) and ATG40 (guide#400). In insets, sequences of sgRNAs are displayed in blue, while ATG codons are in red. **(b,c):** Forced endogenous  $\Delta 40p53$  expression drives Netrin-1 and UNC5B increase. Stable LHCN-M2 cells knockout for p53 gene were generated using firefly luciferase-control (sgLUC), ATG1- (sgp53#10) or ATG40- (sgp53#400) targeting sgRNAs. Western blot analysis, using pan-p53 antibody DO-11, confirmed the complete knockout of all isoforms of p53 in LHCN-M2 cells generated with guide sgp53#400, targeting  $\Delta 40p53$  ATG40 codon, while guide sgp53#10, targeting only ATG1 codon of full-length p53 forced cells to produce a shorter p53 isoform, instead of full-length p53, with a molecular weight corresponding to  $\Delta 40p53$  (b). To reveal endogenous full-length p53 in parental and sgLUC LHCN-M2 cells, a longer exposition is

also shown. Netrin-1, UNC5B and Ku80 protein levels, revealed by western blot, are also displayed. Expression levels of Netrin-1 (NTN1), UNC5B and p21 transcripts were analyzed by quantitative PCR (c). Quantitative PCR results are presented as mean  $\pm$  SD (n=3). ns, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; Ex, exon; MW, molecular weight; KDa, Kilodalton; NTN1, Netrin-1.

**Figure 6.  $\Delta 40p53$  expression sensitizes cells to Netrin-1 interference-induced apoptosis.**

**(a,b):** Parental or stable LHCN-M2 cells, infected with control (sgLUC), full p53 (sgp53#400) or FLp53 only (sgp53#10) knock-out lentiviral constructs, were transfected with scramble (siCTRL) or Netrin-1-specific (siNet) siRNAs and apoptosis induction was evaluated by DNA fragmentation assay (SubG1) (a) or caspase-3 activity (b). DNA fragmentation assay showed in (a) represents a representative experiment (n=3). Caspase-3 activity results in (b) are presented as mean  $\pm$  SD (n=3). \*\*,  $p < 0.01$ ;

**Figure 7. Netrin-1 and  $\Delta 40p53$  gene expression correlates in human tumour biopsies.**

**(a):** Correlation between  $\Delta 40p53$  and Netrin-1 gene expression in melanoma and colorectal cancer biopsies cohort. Expression of  $\Delta 40p53$  was plotted in function of Netrin-1 (NTN1) expression, and goodness of fit was quantified by calculation of the coefficient of determination  $R^2$ . **(b):** Melanoma and colorectal cancer biopsies were divided in

function of relative  $\Delta 40p53$  gene expression, in order to form high (more than median gene expression value) or low  $\Delta 40p53$  expression groups. Netrin-1 gene expression was then evaluated in  $\Delta 40p53$  expression groups. \*,  $p < 0.05$ ; NTN1, Netrin-1; A.U., arbitrary units.

## Supplementary Information

### Supplementary Figure 1.

**(a):**  $\Delta 40p53$ -dependent Netrin-1 induction requires  $\Delta 40p53$  transcriptional activity. Stable A549 cell lines, inducible for wild-type or TAD-mutated ( $\Delta 40p53^{14,15}$ )  $\Delta 40p53$  protein, were treated with Doxycycline. Netrin-1, UNC5B and p21 gene expression was evaluated by quantitative PCR. **(b):** Silencing endogenous p53 expression does not affect  $\Delta 40p53$ -dependent UNC5B up-regulation. Stable A549 cell lines, inducible for  $\Delta 40p53$ , were transfected with a scramble siRNA (siCTRL) or with a specific siRNA targeting exon 2 of p53 gene (sip53). 24 hours after transfection, cells were treated with Doxorubicin or doxycycline for further 24 hours. UNC5B and p21 transcript levels were quantified by quantitative PCR. p53 silencing efficiency was evaluated by quantitative PCR, using full-length p53-specific primers (FLp53), designed in exon 2 of p53 gene. Gene expression was normalized using TATA binding protein (TBP) and beta-glucuronidase (GUSB) genes, used as housekeeping genes. Data are presented as mean  $\pm$  SD (n=3). ns, non-significant; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ . CTRL, not treated, control cells; Dox, doxycycline; DoxoR, doxorubicin; NTN1, Netrin-1; FLp53, full-length p53.

### Supplementary Figure 2.

**(a,b):**  $\Delta 40p53$ -induced Netrin-1 and UNC5B gene expression is not altered by knocking-down endogenous p53. A549 cells were infected using CRISPR/Cas9-modified pLV-hU6-sgRNA-hUbc-dCas9-KRAB-T2a-Puro plasmid, and stable cell lines were generated using firefly luciferase-control (sgLUC) or p53-specific (sgp53A and sgp53B) small guide

RNAs. Endogenous p53 knockout cell lines were then transfected with empty (pITR) or  $\Delta 40p53$  sleeping-beauty plasmids. Stable A549 cell lines knockout for p53 and inducible for  $\Delta 40p53$  were treated with Doxorubicin and Doxycycline for 24 hours. Netrin-1 (a) and UNC5B (b) transcript levels were assessed by quantitative PCR. Data are presented as mean  $\pm$  SD (n=3). \*, p < 0.05; \*\*\*\*, p < 0.0001. CTRL, not treated, control cells; Dox, doxycycline; DoxoR, Doxorubicin; NTN1, Netrin-1; sg, small guide.

### **Supplementary Figure 3.**

Forced endogenous  $\Delta 40p53$  affects GADD45 and BAX gene expression. Stable LHCN-M2 cells knockout for p53 gene were generated using firefly luciferase-control (sgLUC), ATG1- (sgp53#10) or ATG40- (sgp53#400) targeting sgRNAs. Expression levels of Growth Arrest and DNA Damage 45 (GADD45) and BAX transcripts were analyzed by quantitative PCR. Results are presented as mean  $\pm$  SD (n=3). ns, non-significant; \*, p < 0.05.

Figure 1

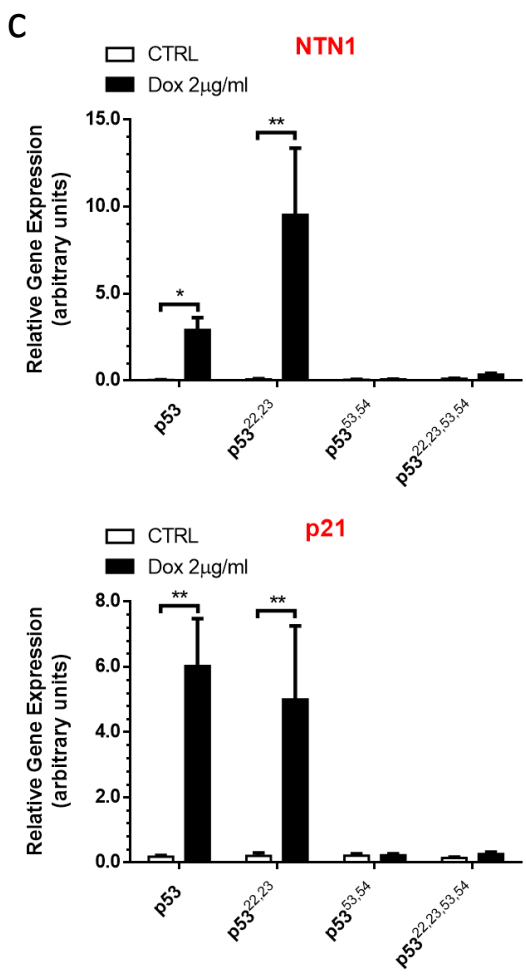
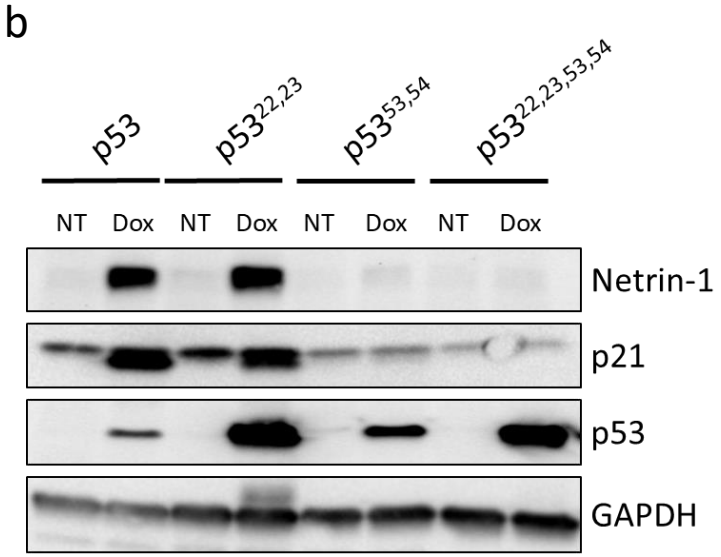
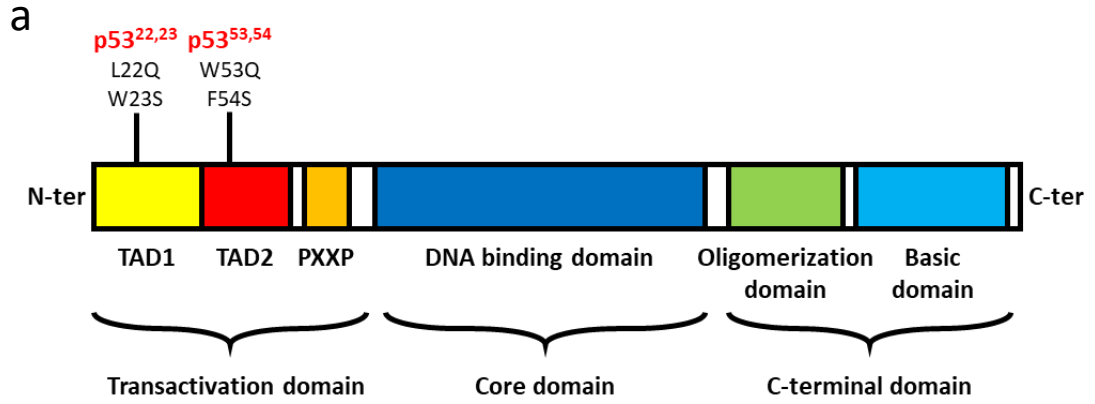


Figure 2

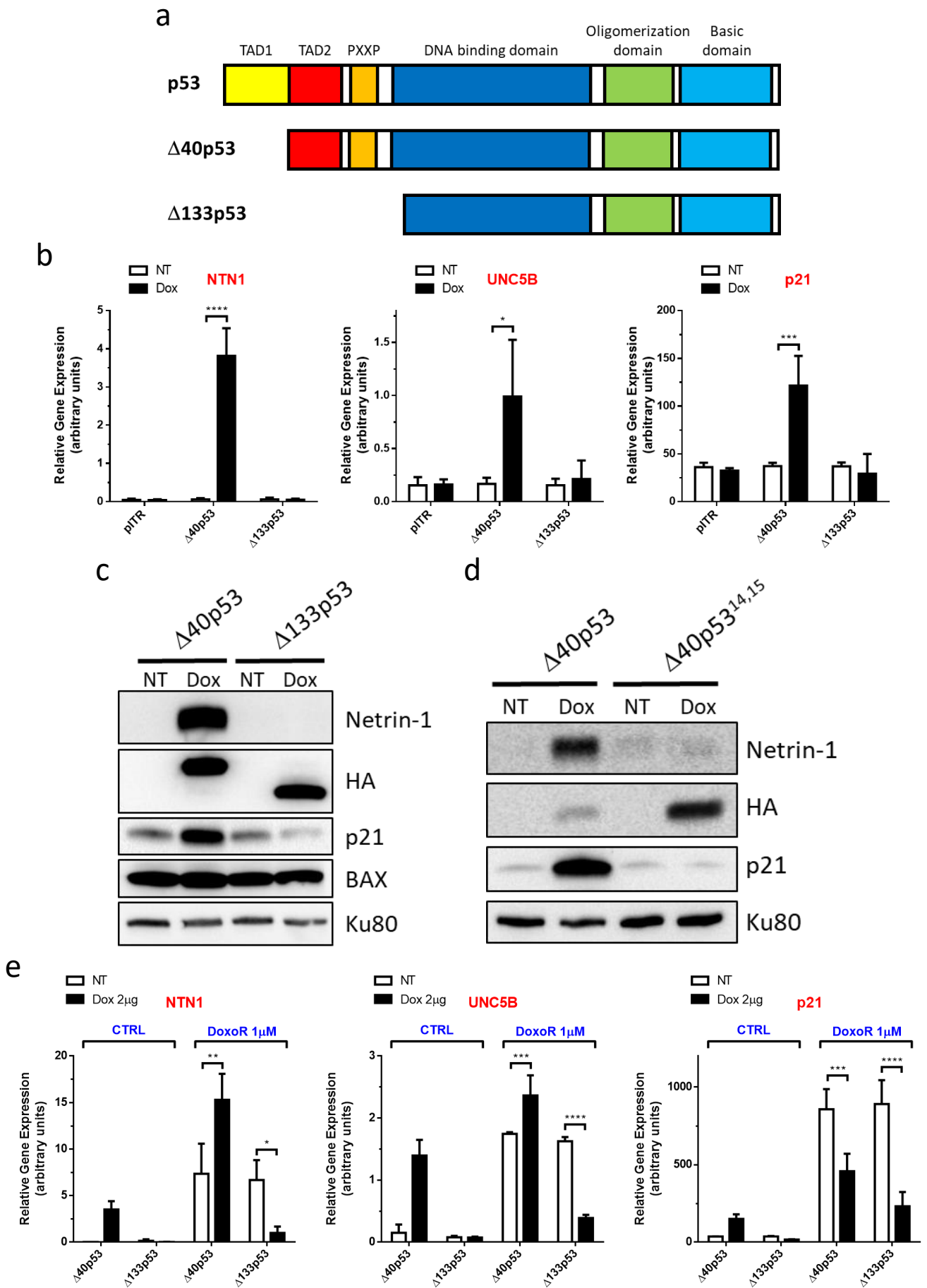


Figure 3

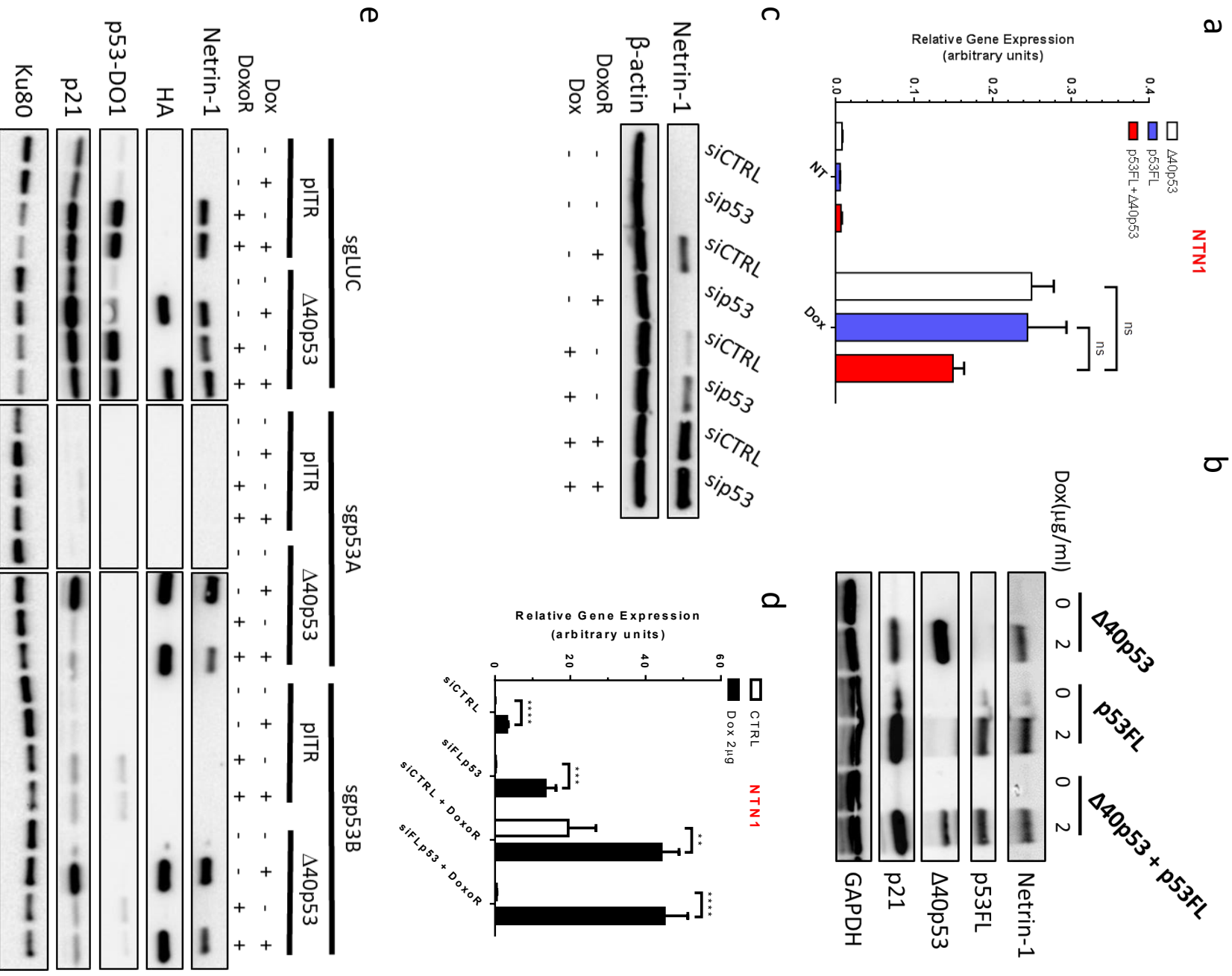




Figure 4

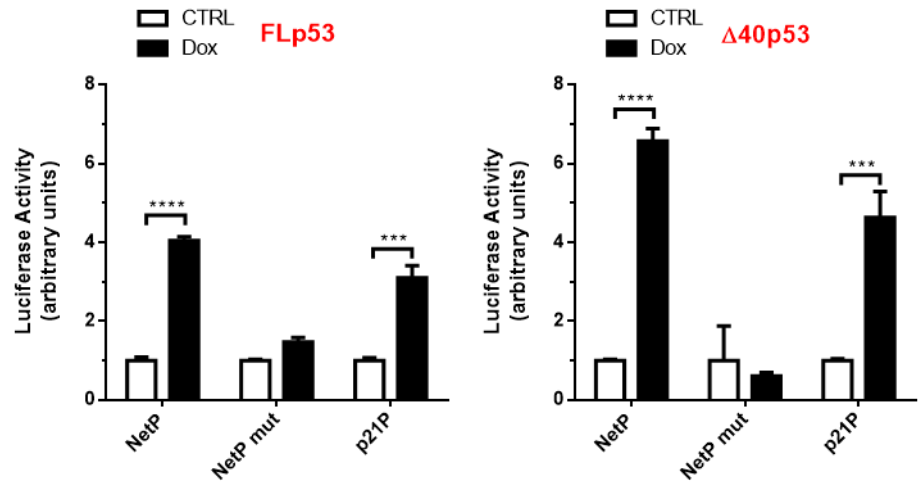
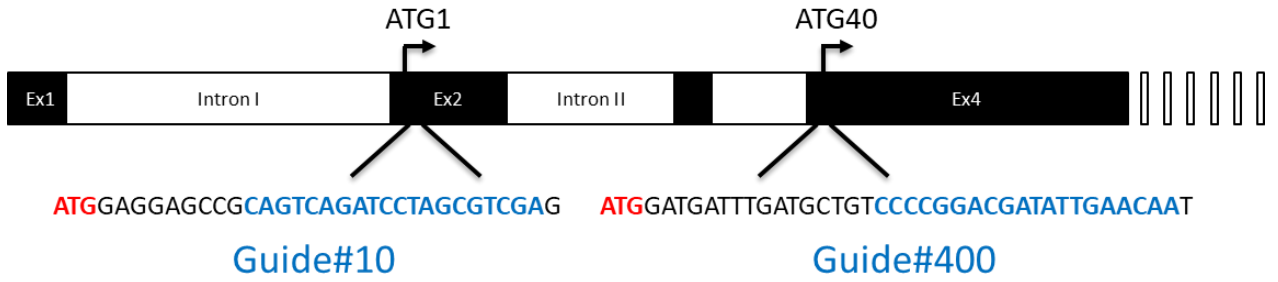
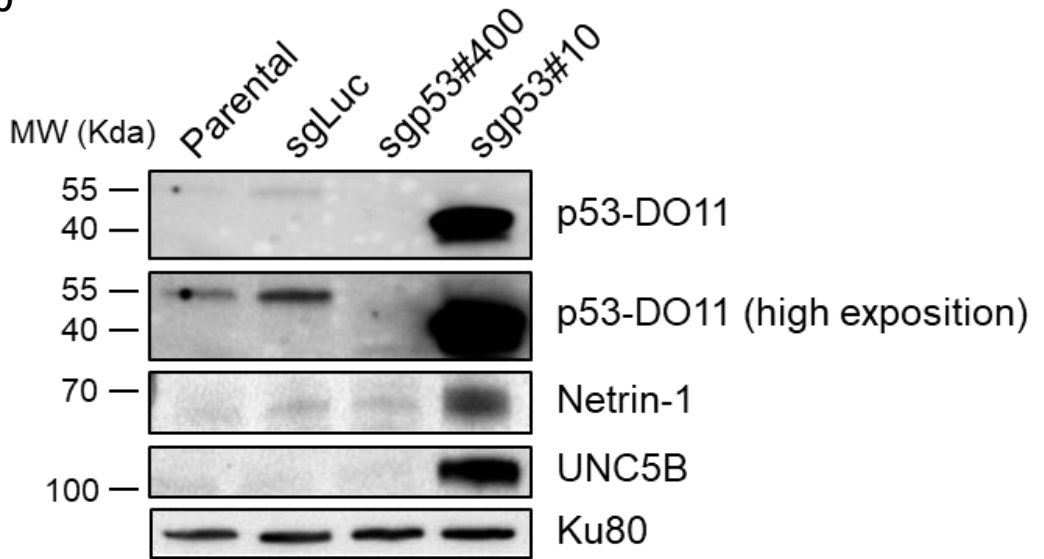


Figure 5

a



b



c

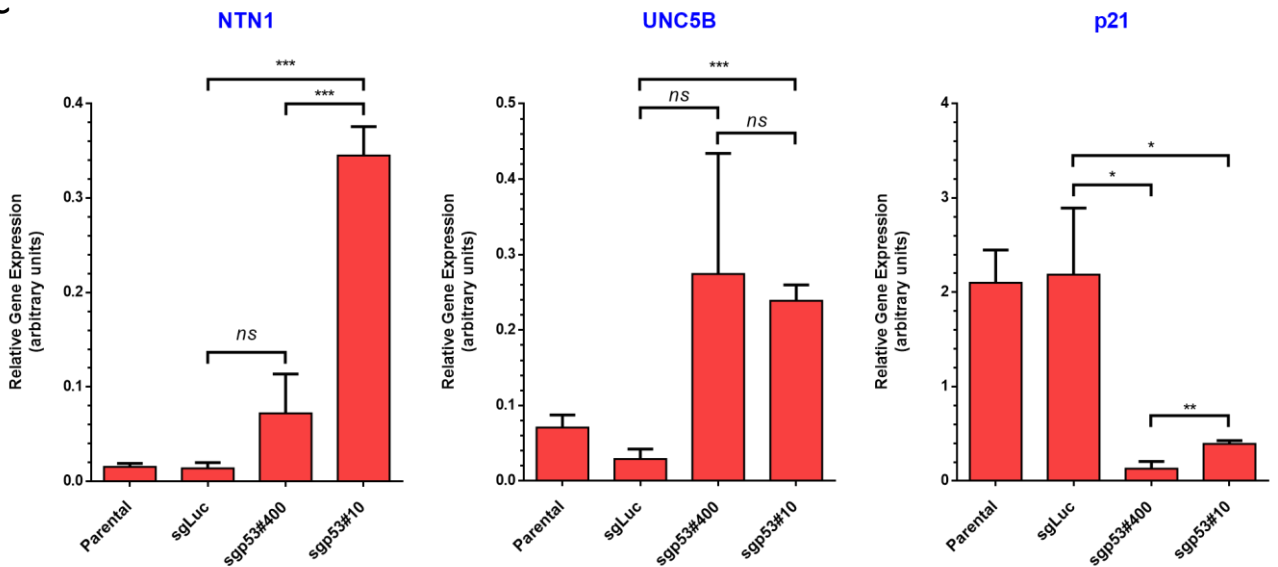


Figure 6

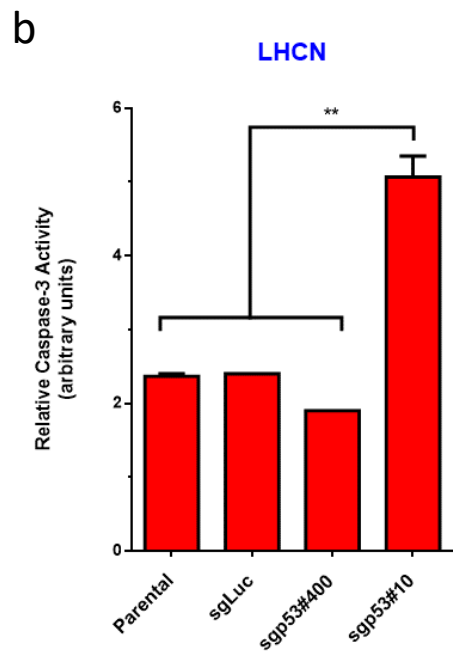
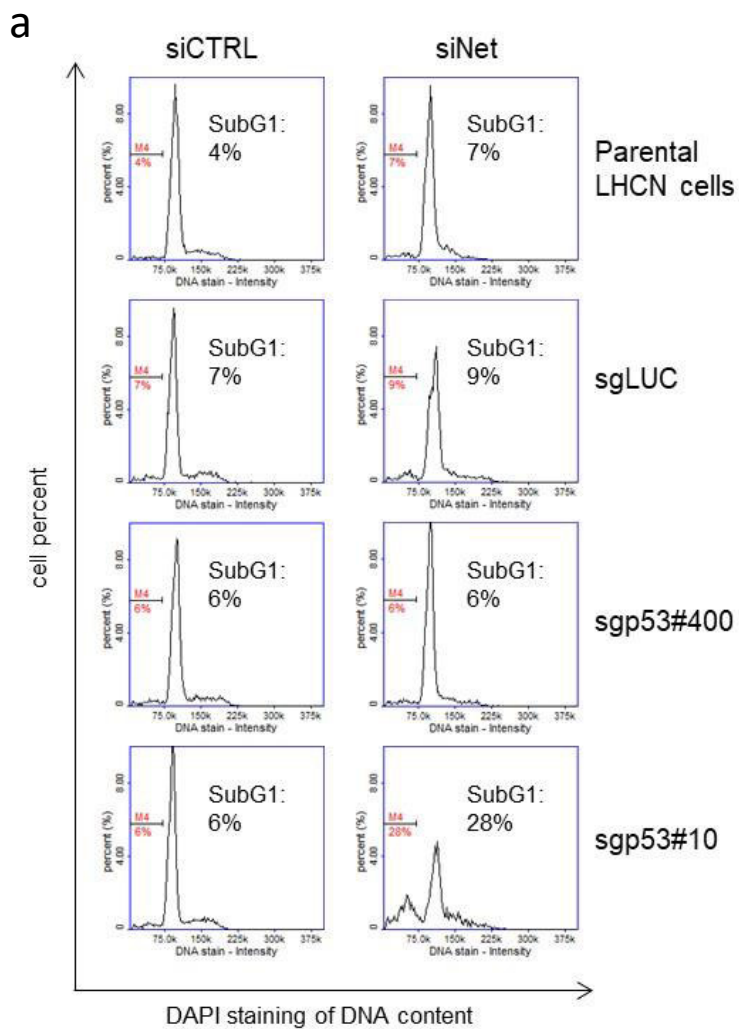
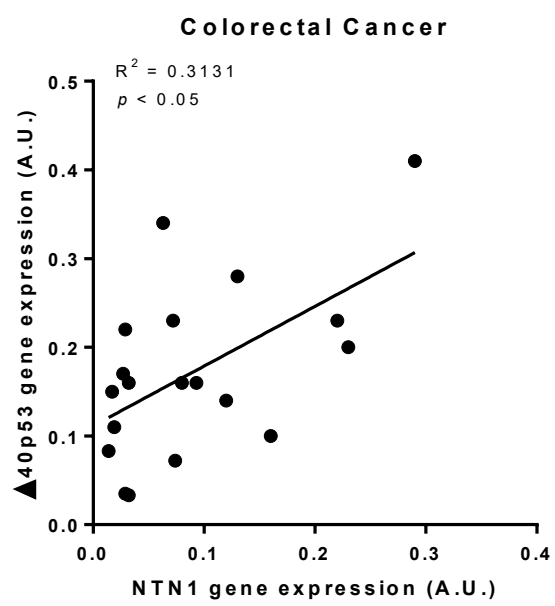
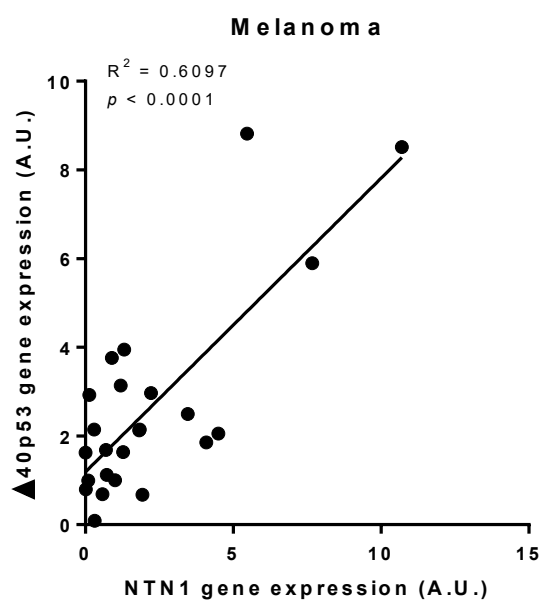
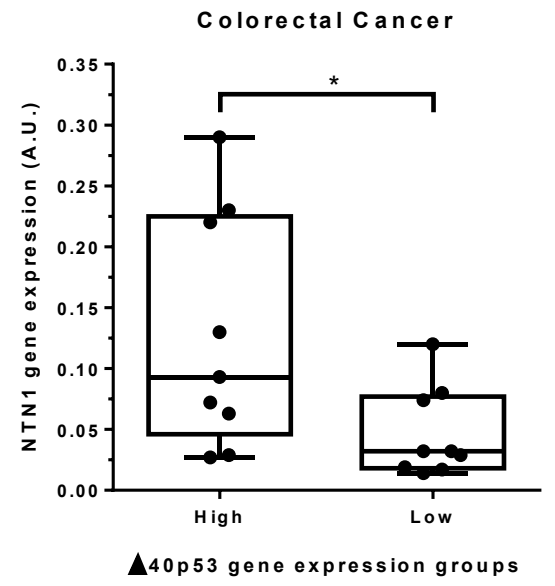
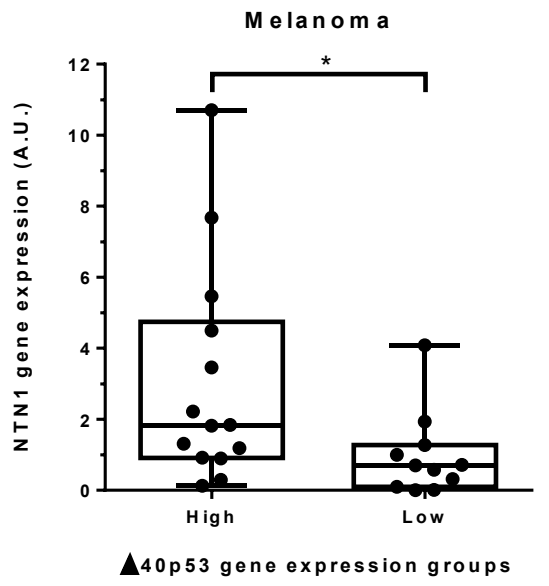


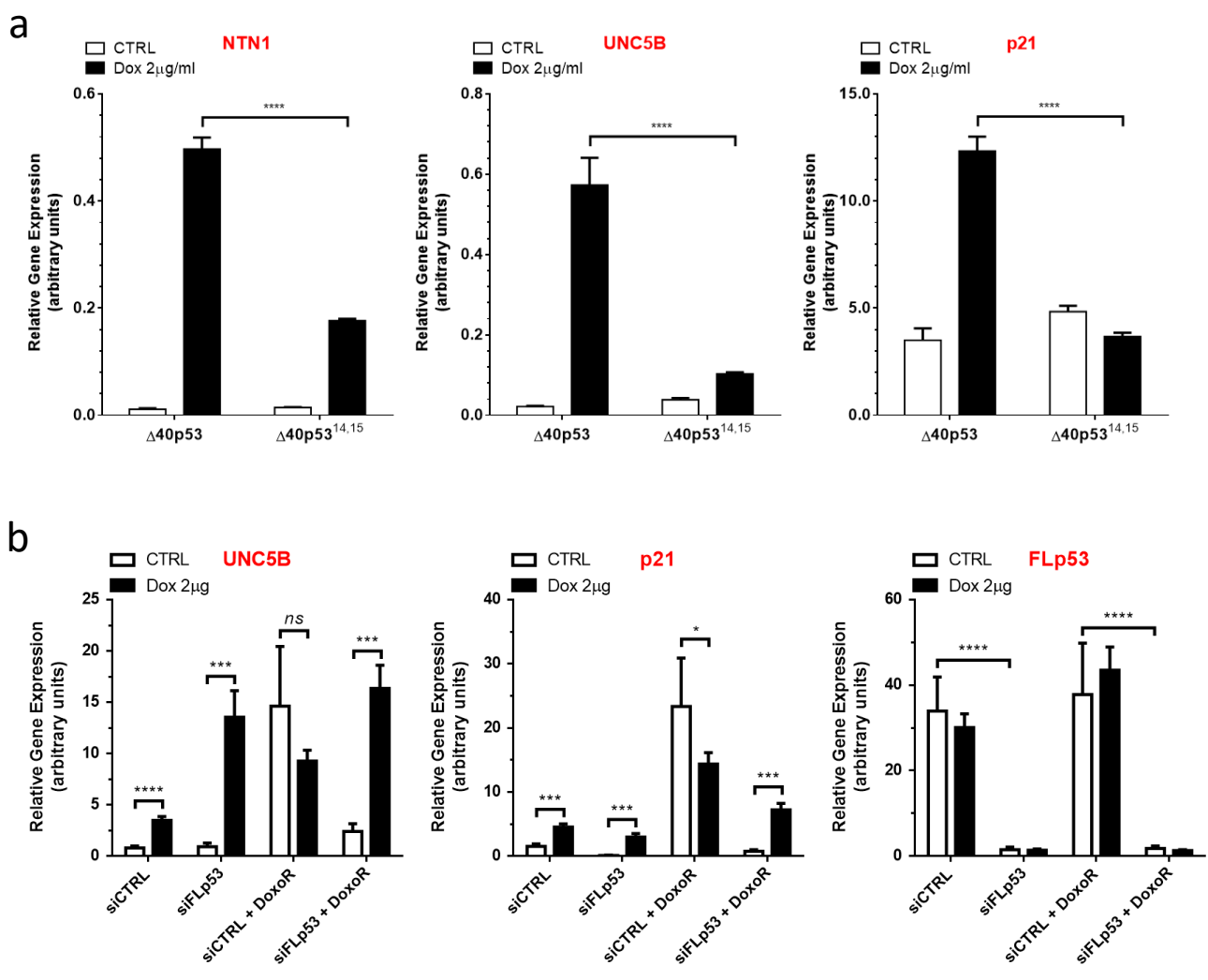
Figure 7

a

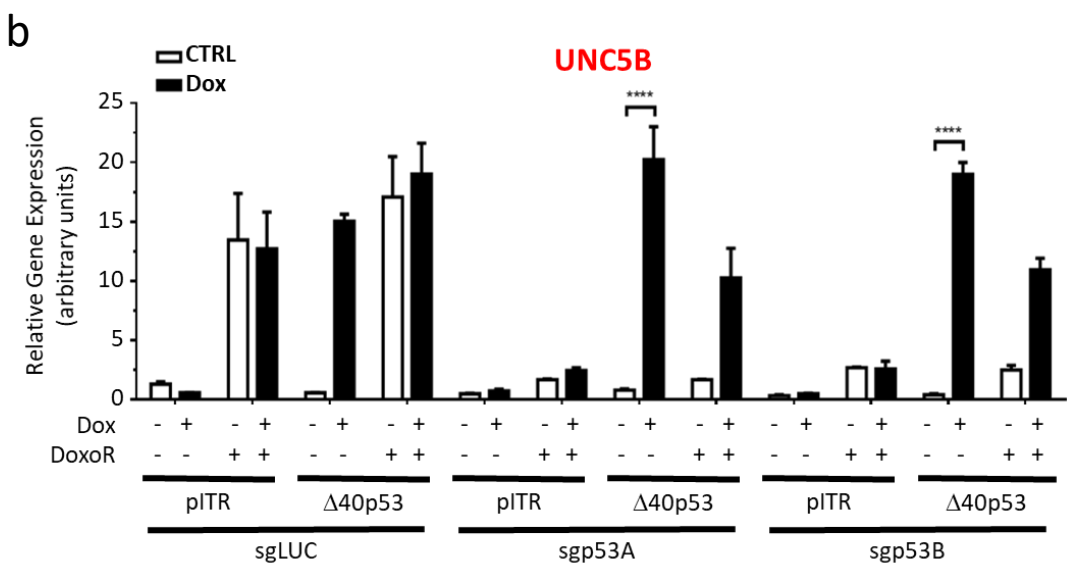
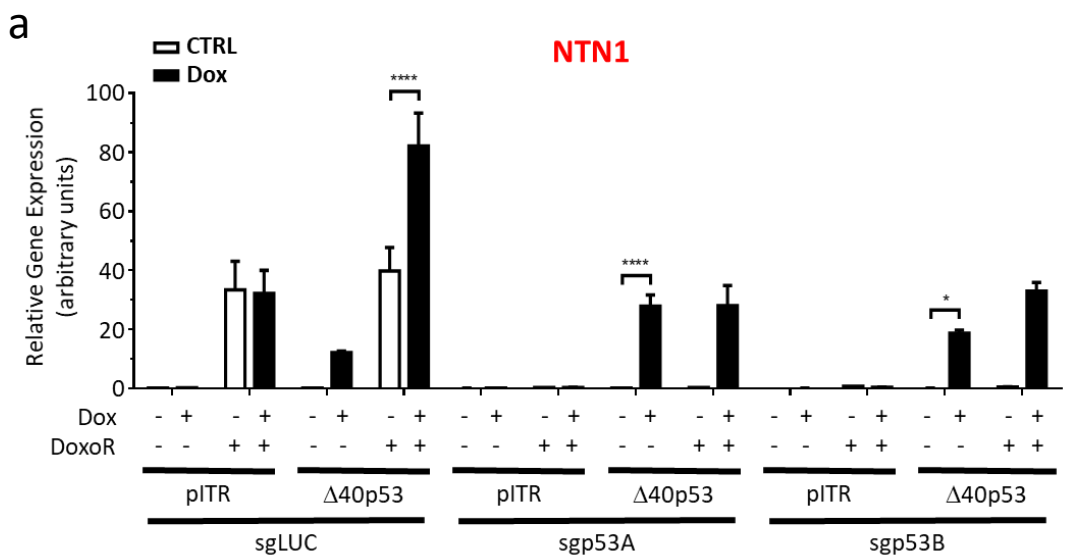


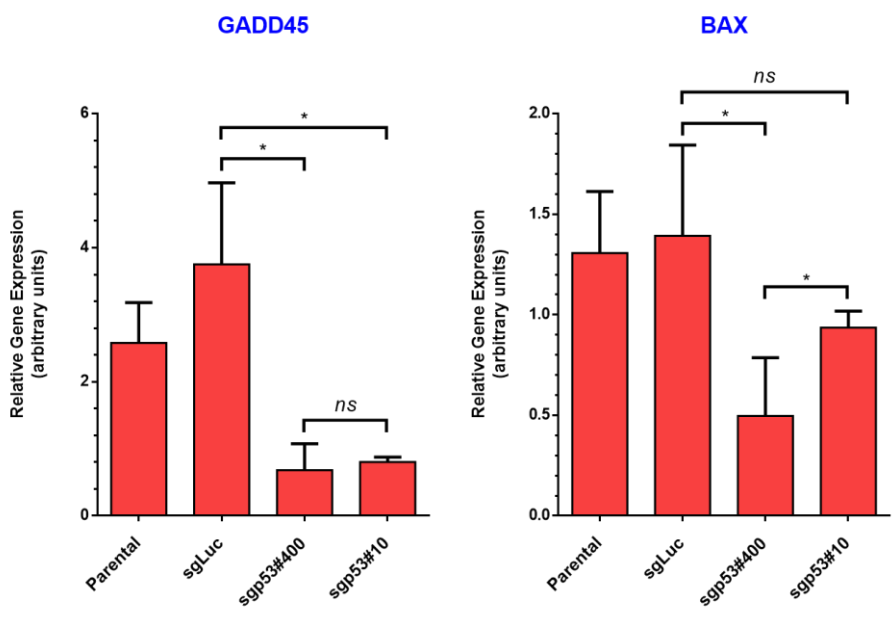
b





Suppl. Figure 2





## **IV. Discussion and Perspectives**



## 6.1 Netrin-1 receptor pathways play an important role in tumorigenesis

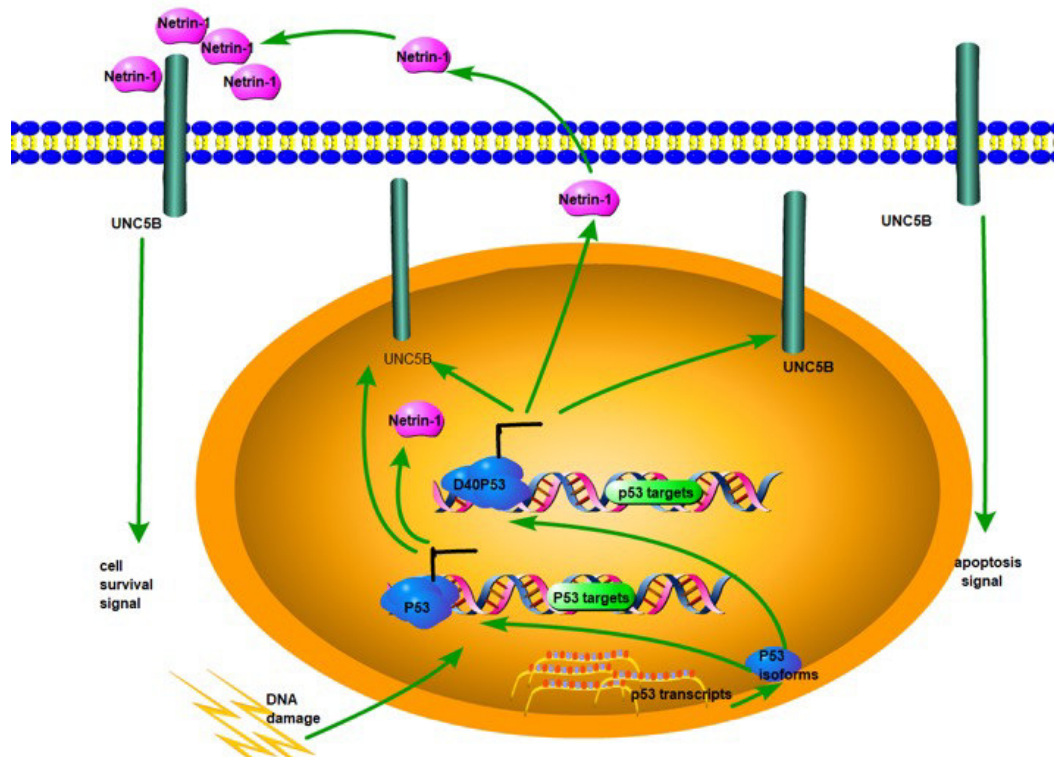
Netrin-1 has been initially shown to play a crucial role in neuronal navigation during nervous system development, mainly through its interaction with its receptors DCC and UNC5H (Serafini et al., 1994). More recently, Netrin-1 and its receptors have been described as modulators of tumorigenesis, and Netrin-1 was observed frequently up-regulated in several aggressive human cancers (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Fitamant et al., 2008; Paradisi et al., 2008). Indeed, Netrin-1 receptors DCC and UNC5H belong to the dependence receptors family, creating survival dependence on their respective ligands by inducing apoptosis when uncoupled by the ligand (Arakawa, 2004; Mehlen and Puisieux, 2006). Netrin-1 pro-tumor function results mainly from its ability to bind dependence receptors DCC and UNC5H, thus inhibiting their pro-apoptotic activity (Castets et al., 2011; Goldschneider and Mehlen, 2010; Llambi et al., 2001; Mazelin et al., 2004; Mehlen et al., 1998). Based on above results, our laboratory identified Netrin-1/UNC5B interaction domains and generated a humanized anti-Netrin-1 antibody that disrupts this interaction and triggers apoptosis of Netrin-1-expressing tumor cells *in vitro* and inhibition of tumor growth and metastasis *in vivo* (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Fitamant et al., 2008; Grandin et al., 2016; Mille et al., 2009; Paradisi et al., 2009). Promisingly, this Netrin-1 neutralizing antibody, called NP137, is currently in Phase I clinical trial in Léon Bérard Cancer Center. However, Netrin-1 inhibition strategy could only be applied in cancer cells expressing Netrin-1. Interestingly, treatment with epigenetic drugs, such as decitabine, increases Netrin-1 gene expression, allowing an efficient combination of NP137 with these drugs to treat tumor showing no or modest Netrin-1 expression (Grandin et al., 2016b). Moreover, in various human cancer cell lines, conventional chemotherapeutic drugs, such as doxorubicin, 5-fluorouracil, taxol and cisplatin, treatments trigger an increase of Netrin-1 and its receptors gene expression (Paradisi et al., 2013). Also, it has been shown that interfering with Netrin-1/receptors interactions sensitizes tumor cells to chemotherapeutic drugs *in vitro* and *in vivo* (Paradisi et al., 2013). In summary, Netrin-1 inhibition strategy is important in developing personalized therapeutic treatments. The knowledge on

how Netrin-1 gene expression is regulated could be then crucial to select patients eligible to anti-Netrin-1 treatment.

## **6.2 $\Delta 40p53$ regulates Netrin-1 and its receptor UNC5B gene expression**

We have recently demonstrated that the transcription factor p53 regulates Netrin-1 and its main receptor UNC5B (Paradisi et al., 2013). Indeed, Netrin-1 promoter contains a p53 binding site in the first intron, responsible for Netrin-1 induction by full-length p53 (FLp53). Here we propose that  $\Delta 40p53$ , a N-terminal truncated isoform of p53, transcriptionally regulates Netrin-1 gene expression, using the same p53 binding site we found in Netrin-1 promoter. More interesting, this regulation seems to be independent by the presence of FLp53, suggesting that  $\Delta 40p53$  can form homo-tetramer in the Netrin-1 promoter (Figure 1). Moreover, we demonstrate that p53 regulation of Netrin-1 gene expression requires transcriptional activity, even if inactivation mutations in the first p53 transactivation domain do not affect Netrin-1 regulation by p53, suggesting a key role of the second transactivation domain. Consequently, deletion of the first transactivation domain, as observed in  $\Delta 40p53$  isoform, is not needed to p53 regulation of Netrin-1 gene expression.

Moreover, we show here that forcing non-tumor cells, like myoblast LHCN cell line, to produce  $\Delta 40p53$  instead of FLp53, is sufficient to increase Netrin-1 and UNC5B gene expression. Indeed, using CRISPR/Cas9 technology, we develop a system to allow deletion of first ATG in p53 gene, driving expression of FLp53, and then force translational machinery to use ATG codon in position 40. In this way, we force LHCN cells to produce  $\Delta 40p53$  at endogenous transcription levels. Moreover, using DNA fragmentation and caspase-3 activity assays we also proved that forced  $\Delta 40p53$  expression amplifies Netrin-1 dependence and sensitizes cells to Netrin-1 interference-induced apoptosis.



**Figure 1**  $\Delta 40p53$  transcriptionally regulate Netrin-1 and its receptor UNC5B gene expression.

### **6.3 Mechanisms involved in $\Delta 40p53$ regulation of Netrin-1 expression**

Early studies pointed-out a role of  $\Delta 40p53$  as a dominant-negative regulator of FLp53 (Marcel et al., 2011). For instance, in human diploid fibroblast WI38 cells, expression of endogenous  $\Delta 40p53\alpha$  increased during the G1/S transition, in parallel with decreased expression of p21. Furthermore, as  $\Delta 40p53\alpha$  lacks the MDM2-binding site, it escapes MDM2-mediated degradation and does not accumulate in response to DNA damage, its expression persisting at low but stable amounts in many cell types (Marcel et al., 2011).

In cancer cell,  $\Delta 40p53$  was described to act as a dominant-negative on FLp53 activity. For instance,  $\Delta 40p53$  itself does not suppress cell viability but it controls p53-mediated growth suppression (Courtois et al., 2002; Vieler and Sanyal, 2018).  $\Delta 40p53$  has a major influence over p53 activity in part through controlling p53 ubiquitination and cell localization:  $\Delta 40p53$  was monoubiquitinated in an MDM2-independent manner, and this was associated with its export out of the nucleus (Ghosh et al., 2004). In the presence of  $\Delta 40p53$ , there was a reduction in MDM2-mediated polyubiquitination and degradation of p53, and this was also associated with increased monoubiquitination and nuclear export of p53 (Ghosh et al., 2004). Another observation revealing  $\Delta 40p53$  negative function on FL-p53 is in serum-starved cells expressing FL-p53.  $\Delta 40p53$  becomes the predominant p53 form during the synchronous progression into S phase after serum stimulation (Courtois et al., 2002). These results suggest that  $\Delta 40p53$  play a role as a transient, negative regulator of p53 during cell cycle progression (Courtois et al., 2002). However, some other papers observed that  $\Delta 40p53$  acts as a positive regulator of FLp53 functions. For instance, in Saos-2 cells,  $\Delta 40p53$  appears to be capable of modulating the stability of FLp53, leading to the persistence in cells of higher levels of FLp53 bearing phosphoserine 15, a hall-mark of p53 activation (Hafsi et al., 2013). Dramatic effects have been shown when both FLp53 and  $\Delta 40p53$  are co-expressed, and these effects are consistent with an increase and modulation of p53-dependent suppressive effects rather than an inhibition of these effects (Davidson et al., 2010; Maier et al., 2004; Pehar et al., 2010). Indeed, in melanoma

cells,  $\Delta 40p53$  induces apoptosis, but only in cells expressing wild-type FLp53 (Takahashi et al., 2014). The contradictory functions of  $\Delta 40p53$  isoform have been explained by dose-dependent effect of hetero-oligomerization of p53 isoforms, dependent on the relative expression levels (Anbarasan and Bourdon, 2019). Co-expression of the two proteins  $\Delta 40p53$  and FLp53 induce a decrease in the transcriptional activity, but the amplitude of the effect varied depending upon the predicted composition of the hetero-tetramer. It was reported no decrease and possibly a small increase in transcriptional activity for hetero-tetramers containing 1 or 2 monomers of  $\Delta 40p53$  in  $\Delta 40p53/FLp53$  complex, and a strong decrease for hetero-tetramers containing 3 monomers of  $\Delta 40p53$  (Hafsi et al., 2013).

Here, our results show that  $\Delta 40p53$  can regulate Netrin-1 expression. As Netrin-1 is also regulated by FLp53 (Paradisi et al., 2013), its regulation by  $\Delta 40p53$  expression, finely tuned by inducible sleeping beauty system we used, could be barely due to p53 stabilization and activation by hetero-oligomers  $\Delta 40p53/FLp53$ . However, our results, showing Netrin-1 up-regulation by  $\Delta 40p53$  in H1299 p53-null cells or upon FLp53 knock-out in A549 p53-wild-type cells, suggest the formation of a  $\Delta 40p53$ -only homo-tetramer, binding and activating Netrin-1 promoter. This is also confirmed by our experiments with skeletal myoblast LHCN cells. Indeed, using CRISPR/Cas9 technology and a particular set of sgRNAs, we forced endogenous expression of  $\Delta 40p53$ , instead of FLp53, thus triggering Netrin-1 gene expression. As LHCN cells expressing endogenous  $\Delta 40p53$  do not express FLp53, we confirm that Netrin-1 regulation by  $\Delta 40p53$  is independent of FLp53. On the other hand, binding of  $\Delta 40p53$  to p53 responsive elements located in MDM2, GADD45 and BAX genes, leading to FLp53-independent transactivation, has been already reported (Yin et al., 2002).

## **6.4 $\Delta 40p53$ involved in controlling pluripotency in embryonic stem cells (ESCs)**

Besides high expression in human cancers,  $\Delta 40p53$  isoform has been recently involved in controlling pluripotency in embryonic stem cells (ESCs). A recent study by Ungewitter and Scrable has determined that  $\Delta 40p53$  was the major p53 isoform expressed in mouse embryonic stem cells (ESC) as well as during the early stages of embryogenesis (Ungewitter and Scrable, 2010). This isoform is highly expressed in ESCs, regulating progression from pluripotency and differentiation in these cells. Data showing altering the dose of  $\Delta 40p53$  had a strong impact on the maintenance of the ESC state. Haploinsufficiency for  $\Delta 40p53$  (lower level of this isoform), caused loss of pluripotency and changes in cell-cycle with the acquisition of a cell-cycle profile characteristic of the one of somatic, differentiated cells. By contrast, increased dosage of  $\Delta 40p53$  caused an extension of pluripotency and prevented progression to a more differentiated state. These observations suggest that in mouse embryonic cells, high levels of expression of  $\Delta 40p53$  prevent the activation of a mechanism of exit from ESC status into a somatic cell status (Ungewitter and Scrable, 2010).

Moreover,  $\Delta 40p53$  inhibits loss of pluripotency associated with FLp53 activation, and promotes ESCs survival. It has been proposed that  $\Delta 40p53$  isoform role in ESCs is merely to suppress FLp53 activity, allowing pluripotency transcription factors to accumulate. (Ungewitter and Scrable, 2010). Interesting, Netrin-1 has also been involved in pluripotency control in ESCs. A recent paper showed that Netrin-1 expression enhances mouse ESCs self-renewal, by blocking UNC5B-induced cell death. Moreover, Netrin-1 regulates induced pluripotent stem (iPS) cell generation and pluripotency maintenance (Ozmadenci et al., 2015). Together with our results showing a direct regulation of Netrin-1 by  $\Delta 40p53$ , these data could suggest that  $\Delta 40p53$  promotes ESCs survival by regulating Netrin-1 expression, allowing regulation of pluripotency. Of course, more detailed data is needed to confirm this conclusion.

## **6.5 Regulation of Netrin-1 and UNC5B by $\Delta 40p53$ isoform have significant therapeutic consequence**

p53 isoforms are differentially expressed in several human cancers (Khoury and Bourdon, 2010; Vieler and Sanyal, 2018). For example,  $\Delta 40p53$  results up-regulated in tumor breast tissue, compared to normal breast, and associated with aggressive triple-negative breast cancer (Avery-Kiejda et al., 2014), as well as in mucinous ovarian cancer (Hofstetter et al., 2012). Moreover,  $\Delta 40p53$  isoform is well-expressed in melanoma cell lines and biopsies, while undetectable in melanocytes and normal tissue (Avery-Kiejda et al., 2008). However, high expression and cancer aggressiveness correlated to  $\Delta 40p53$  has been associated to dominant-negative function of this isoform on FLp53 activity. Interesting, we show here a positive correlation between  $\Delta 40p53$  and Netrin-1 gene expression in melanoma and colorectal cancer cohorts. Moreover, Netrin-1 and its dependence receptors have been involved in melanoma and colorectal cancer progression (Bernet et al., 2007; Boussouar et al., 2019; Castets et al., 2011; Paradisi et al., 2008). Then, our data could suggest for the first time a FLp53-independent pro-tumor role of  $\Delta 40p53$ , since its ability to transactivate an anti-apoptotic protein such as Netrin-1.

Regulation of Netrin-1 and UNC5B receptor by  $\Delta 40p53$  isoform could have significant therapeutic consequence. Indeed, the fact that both the ligand and its dependence receptor are up-regulated in human cancer co-expressing  $\Delta 40p53$  could enhance dependence of tumor cell survival to Netrin-1 expression. Specifically, inhibition or interference of Netrin-1 in these cancer cells should lead to dependence receptor-induced apoptosis and then tumor regression. Our results demonstrate that silencing of Netrin-1 expression by RNA interference induces cell death only in cells that are forced to produce  $\Delta 40p53$  and consequently express high levels of Netrin-1 and UNC5B. From a therapeutic point of view, this information suggests that interference of the binding between Netrin-1 and UNC5B with a more therapeutic relevant treatment, such as anti-Netrin-1 neutralizing antibody (Grandin et al., 2016) we developed in our laboratory, should be more efficient in human tumors expressing high levels of  $\Delta 40p53$ .

In conclusion, we show here for the first time that  $\Delta 40p53$  isoform could positively regulate an anti-apoptotic protein, such as Netrin-1, in a FLp53-independent way. In addition, this regulation, achieved by both over-expressed or endogenous  $\Delta 40p53$ , could explain increased transcript levels of this protein, observed in several human cancers. Indeed, each mutation changing the ratio between FLp53 and  $\Delta 40p53$  could confer a selective advantage to cancer cells. Of course,  $\Delta 40p53$  could inhibit tumor suppressor activity of FLp53, acting as a dominant-negative protein, however it could also actively transactivate pro-tumor factor, such as Netrin-1, as our results seem to suggest. In this situation, a promising therapeutic strategy could be to inhibit pro-tumor factor activity, as we propose for Netrin-1, instead of targeting dominant-negative function of  $\Delta 40p53$ .



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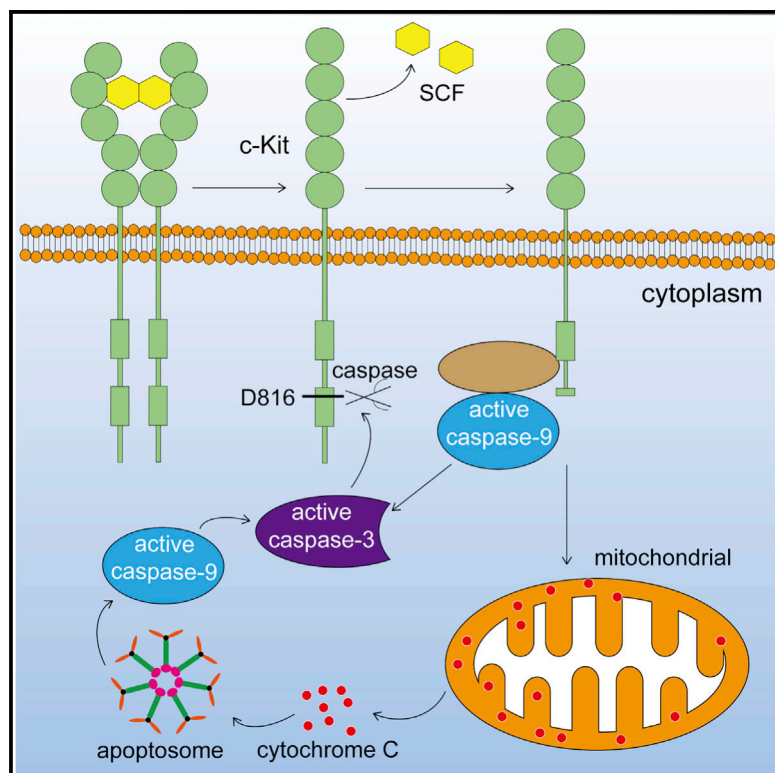


## **VI. Annexes**

# Molecular Cell

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

### Graphical Abstract



### Authors

Hong Wang, Amina Boussouar,  
Laetitia Mazelin, ...,  
David Goldschneider, Andrea Paradisi,  
Patrick Mehlen

### Correspondence

andrea.paradisi@lyon.unicancer.fr (A.P.),  
patrick.mehlen@lyon.unicancer.fr (P.M.)

### 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

Hong Wang,<sup>1</sup> Amina Boussouar,<sup>1,4</sup> Laetitia Mazelin,<sup>1,4</sup> Servane Tauszig-Delamasure,<sup>1,4</sup> Yan Sun,<sup>1</sup> David Goldschneider,<sup>1,2</sup> Andrea Paradisi,<sup>1,5,\*</sup> and Patrick Mehlen<sup>1,2,3,5,6,\*</sup>

<sup>1</sup>Apoptosis, Cancer and Development Laboratory – Equipe labellisée “La Ligue,” LabEx DEVweCAN, Centre de Recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Université de Lyon, Université Claude Bernard Lyon1, Centre Léon Bérard, 69008 Lyon, France

<sup>2</sup>Netris Pharma, 69008 Lyon, France

<sup>3</sup>Department of Translational Research and Innovation, Centre Léon Bérard, 69008 Lyon, France

<sup>4</sup>These authors contributed equally

<sup>5</sup>Senior author

<sup>6</sup>Lead Contact

\*Correspondence: [andrea.paradisi@lyon.unicancer.fr](mailto:andrea.paradisi@lyon.unicancer.fr) (A.P.), [patrick.mehlen@lyon.unicancer.fr](mailto:patrick.mehlen@lyon.unicancer.fr) (P.M.)

<https://doi.org/10.1016/j.molcel.2018.08.040>

## 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 Homolog (SHH) 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 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 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 Rönnstrand, 2012). In other tumors, such as neuroblastoma, the role of c-Kit function is still controversial. Although some reports showed no correlation between c-Kit and SCF status and clinical progression, 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

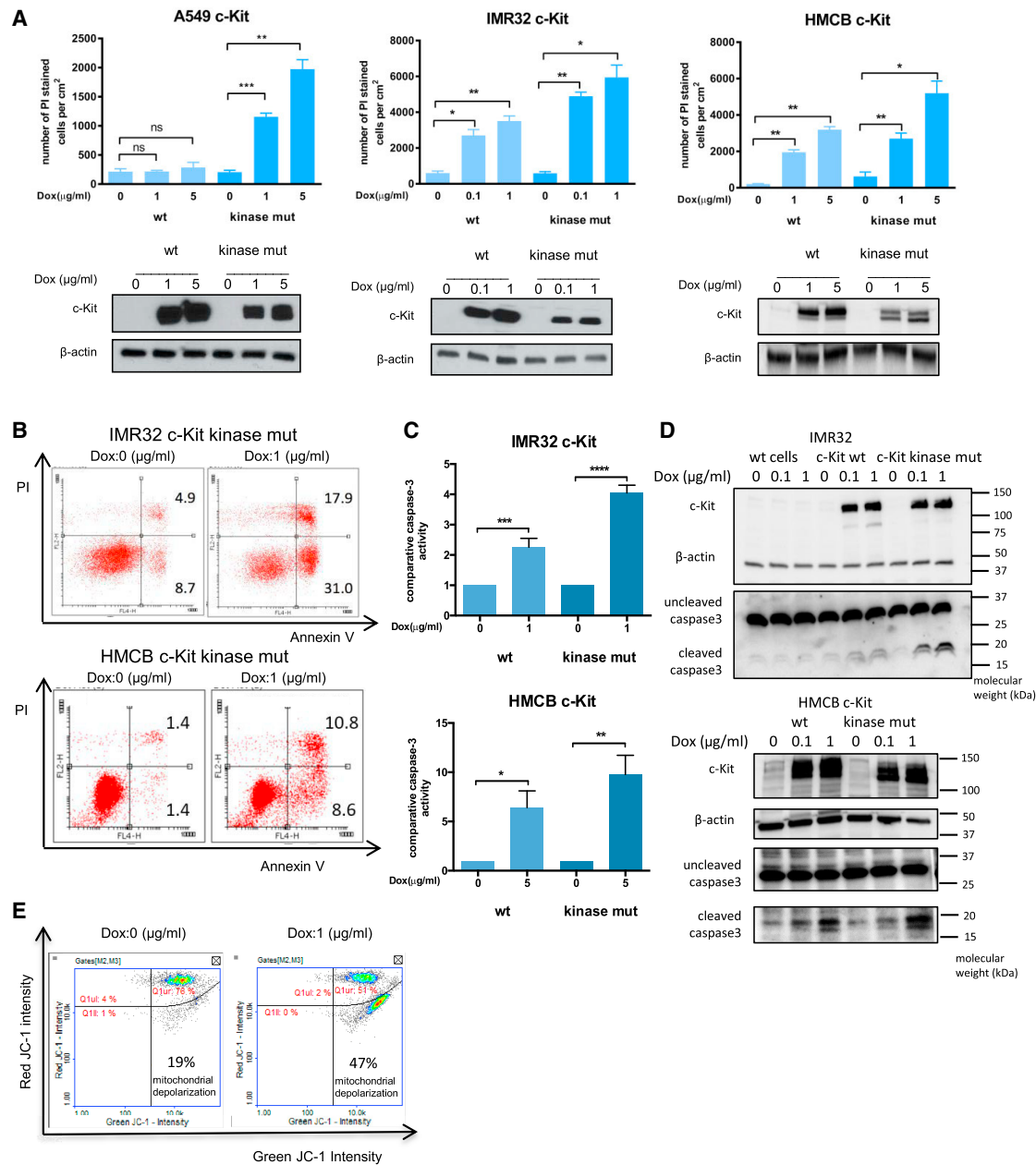
To study the effect of c-Kit on cell death, we generated several stable cell lines in which c-Kit expression is induced by adding doxycycline in the culture medium. To avoid positive signaling (i.e., kinase activation) that may induce survival signals, we generated kinase-mutated c-Kit-expressing cell 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 (Figures 1A and 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 [HMGB]) 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 HMGB 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 HMGB 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 HMGB 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-penta-noic 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 HMGB 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 HMGB 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 HMGB 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).

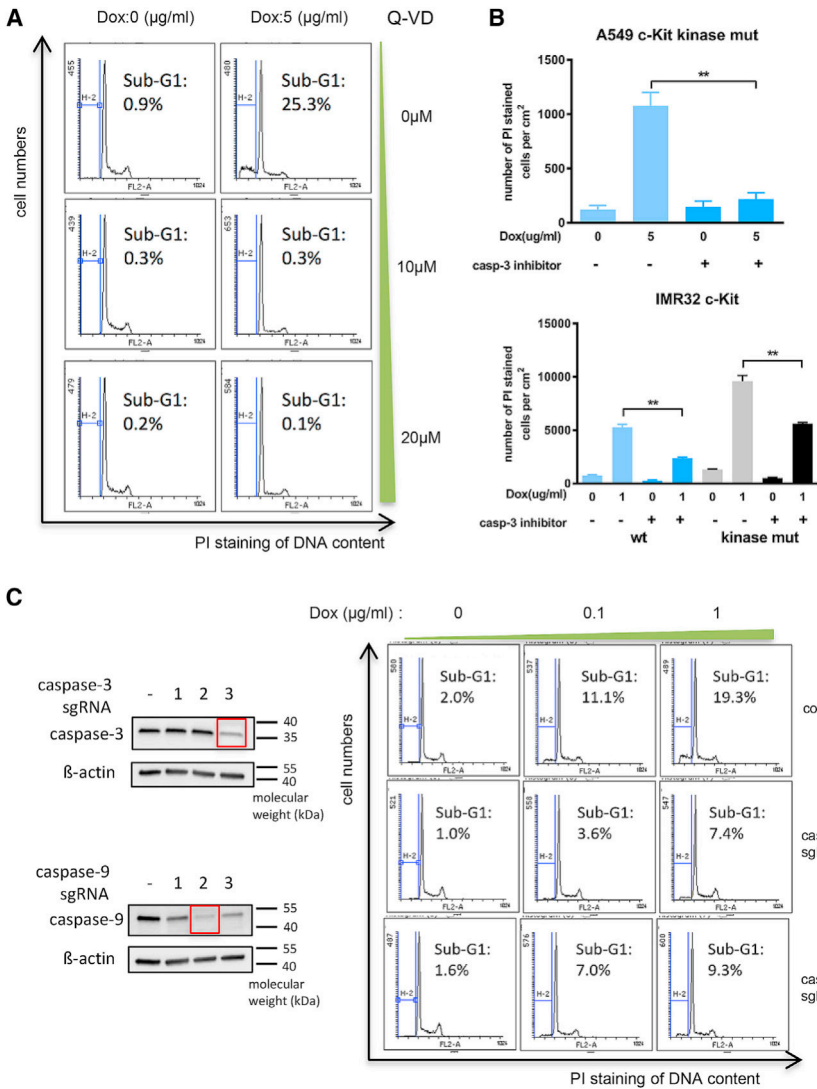
(B) c-Kit-induced cell death is associated with phosphatidylserine flipping to the extracellular surface of the cell, a specific hallmark of apoptotic cell death (Vermes et al., 1995). IMR32 and HMCB stable cell lines were treated with Dox, and the apoptosis rate was quantified by AnnexinV and PI staining 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).

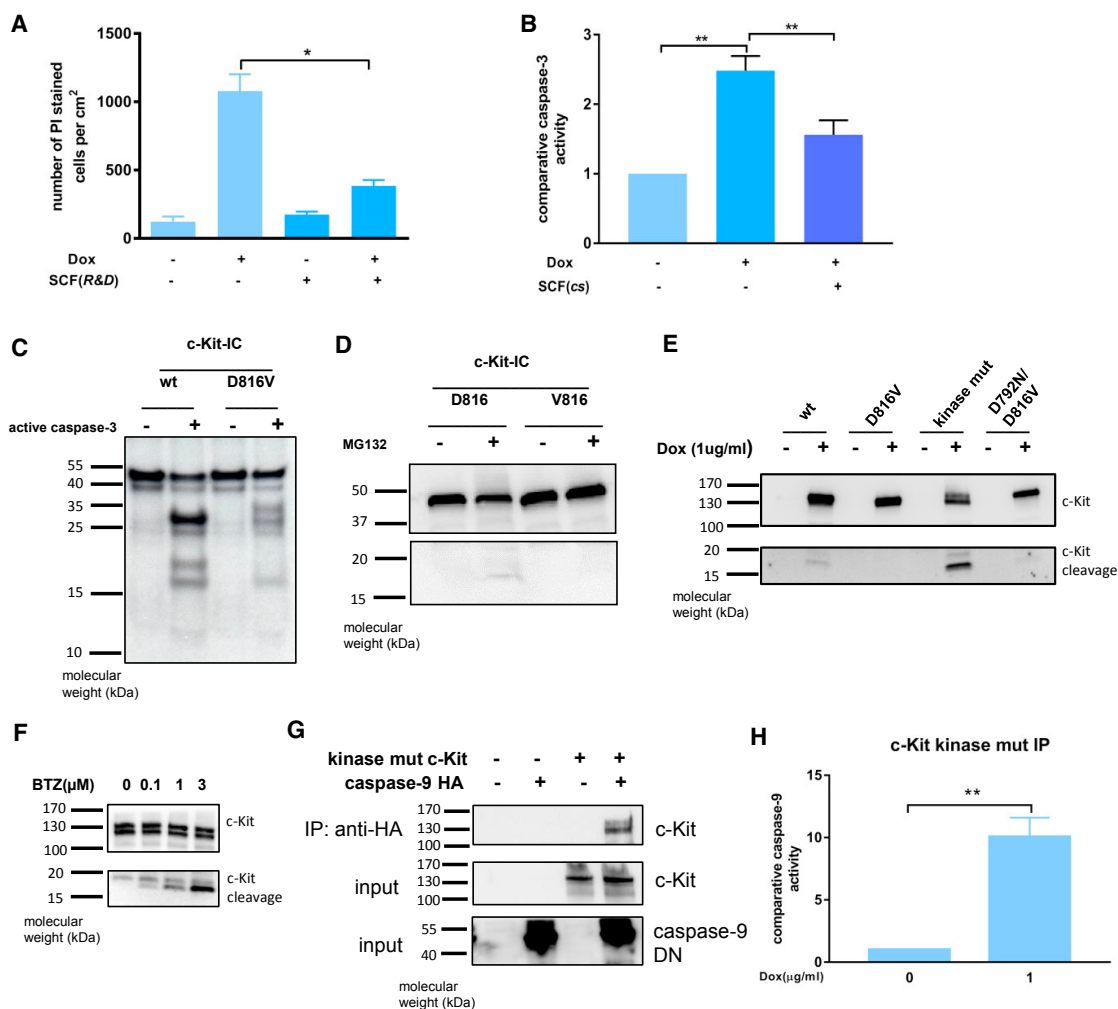
Data are representative of at least three replicates as mean ± SEM. \*\*p < 0.01.

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

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



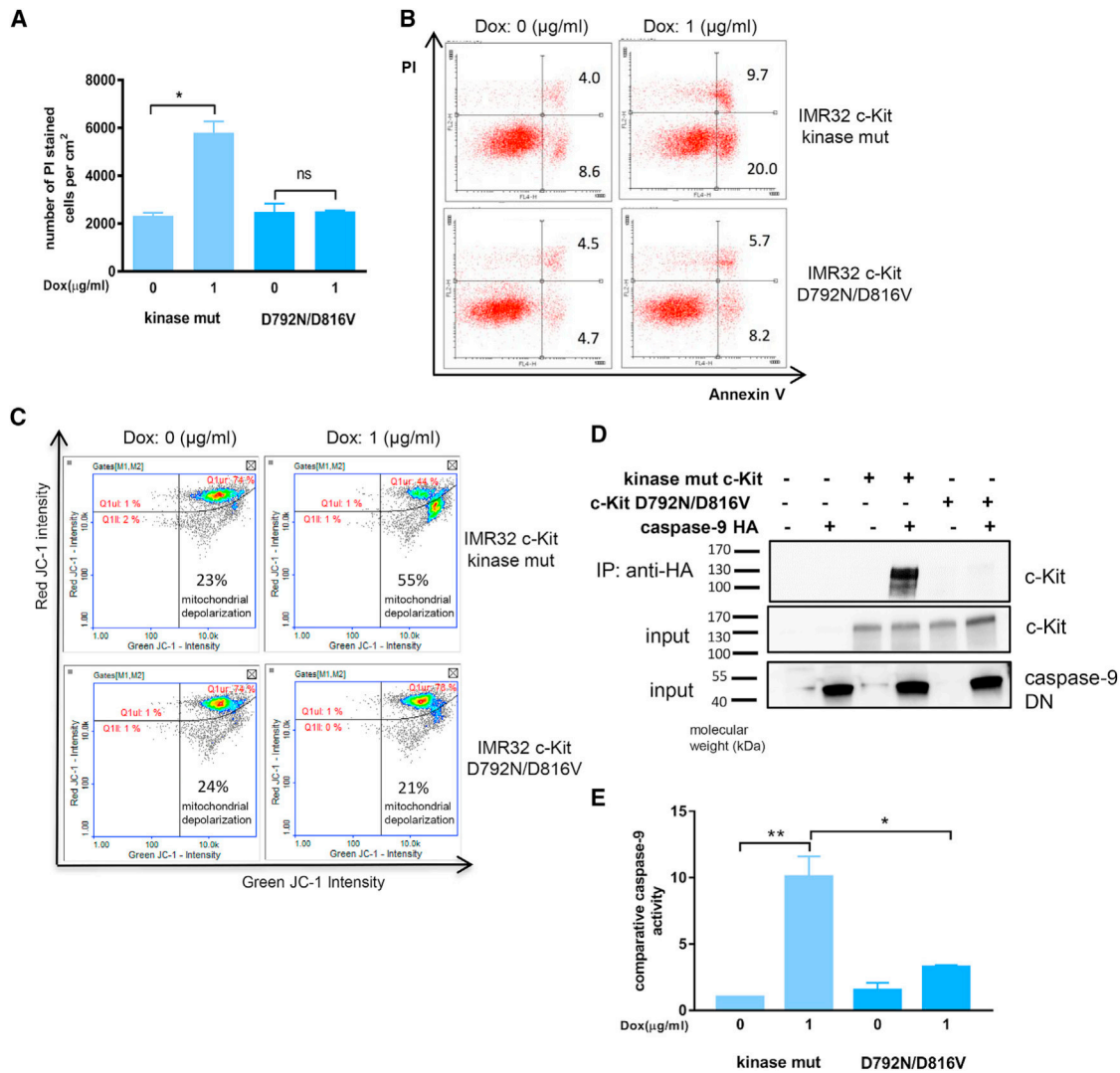
**Figure 3. c-Kit-Induced Apoptosis Is Inhibited by SCF and Is Mediated by Intracellular Caspase-like Cleavage Driving a Caspase Activation Amplification Loop**

(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**

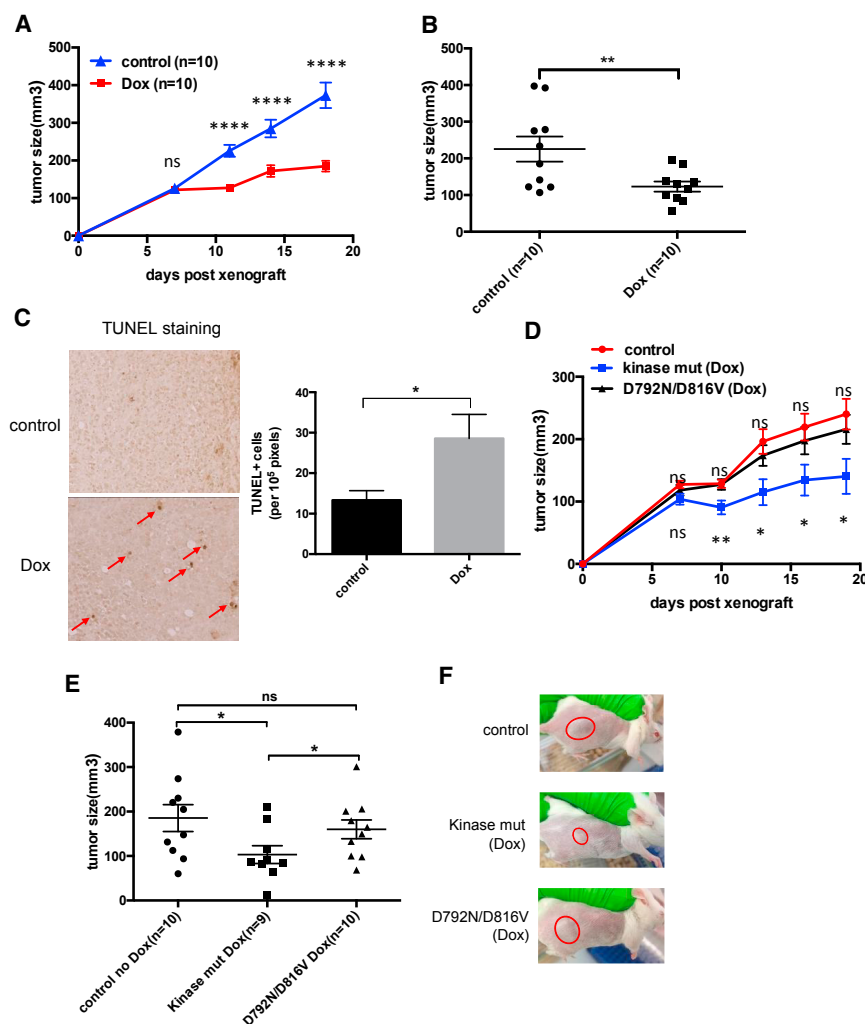
(A–C) c-Kit caspase cleavage is required for cell death induction. The IMR32 stable cell line inducible for kinase-mut or kinase- and caspase-mutated (D792N/D816V) c-Kit was treated with the indicated Dox concentrations. Apoptotic cell death was then evaluated by PI staining 36 hr after Dox treatment 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.





### 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).

### 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.

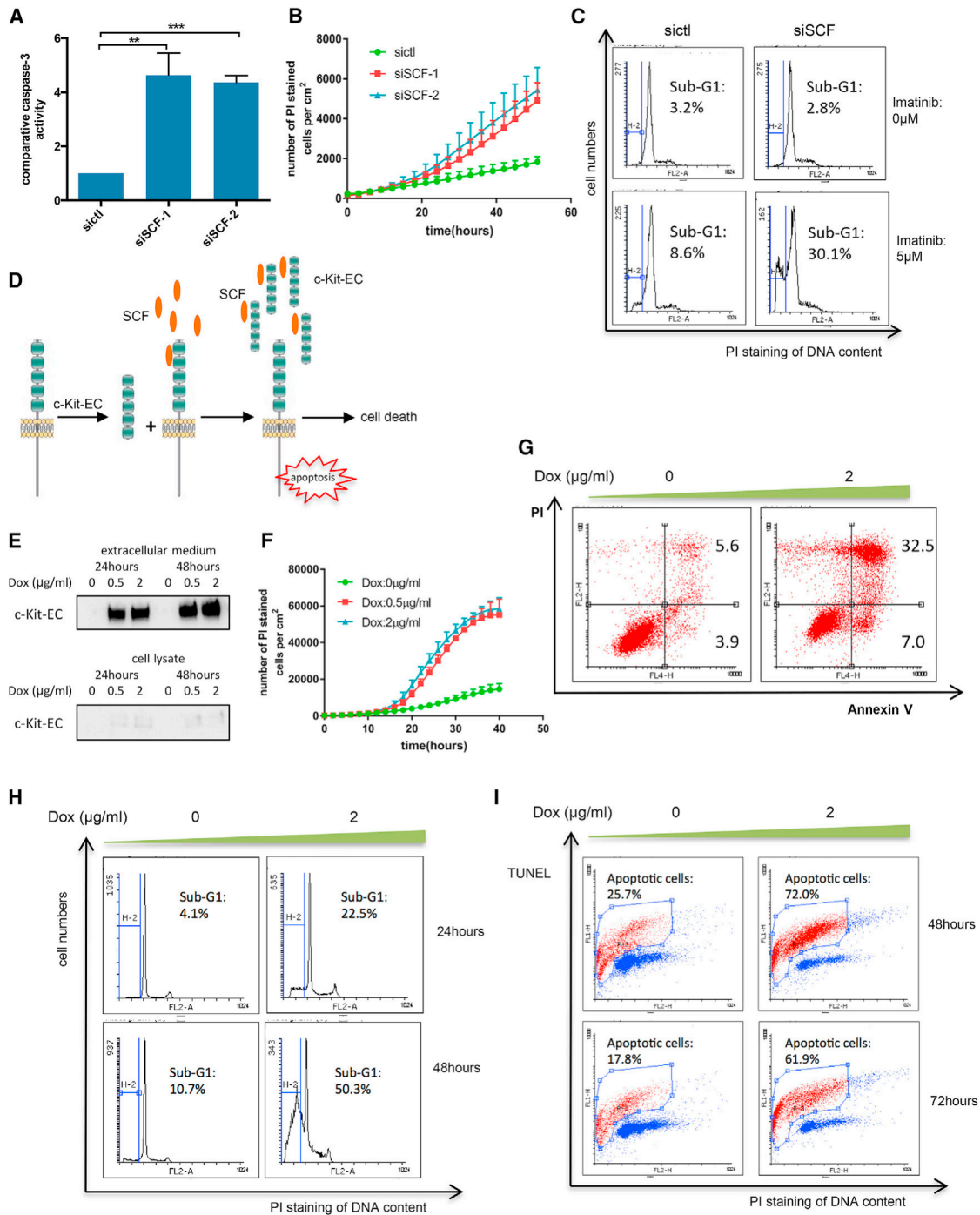
Data are representative as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

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 Rönstrand, 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.

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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

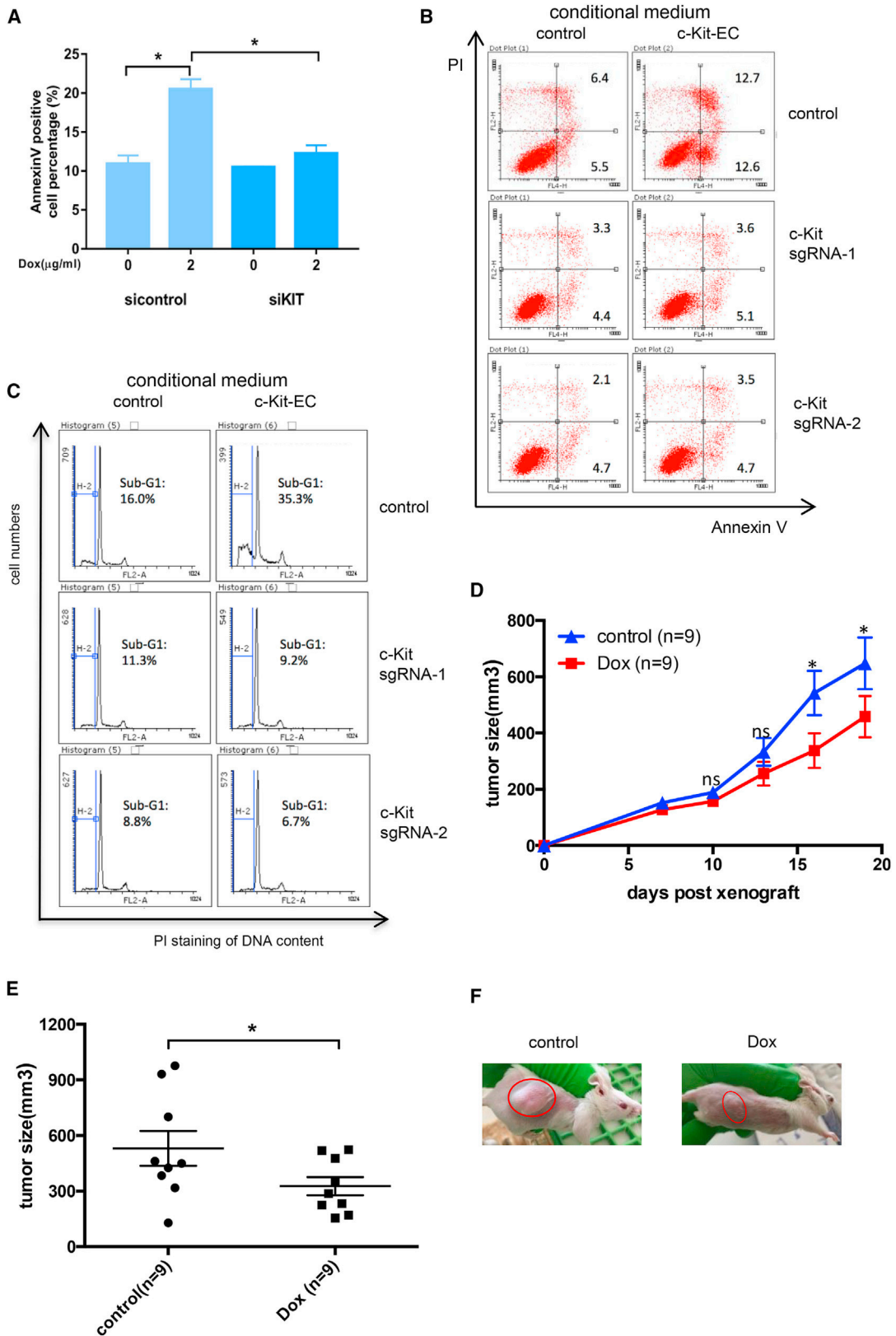
The proto-oncogene c-Kit has been classically considered only as a receptor tyrosine kinase, and most of the studies on c-Kit focused on its kinase activity. Hence, therapeutic strategies targeting c-Kit have been centered on inhibition of its kinase activity, as exemplified by the development of imatinib (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.



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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; Thiebault 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-Rodríguez 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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  - Animal model
- METHOD DETAILS
  - Transfection Procedures, Reagents
  - Generation of stable cell lines
  - Quantitative RT-PCR
  - Western Blotting Analysis
  - Membrane staining of wild-type or mutated c-Kit
  - *In Vitro* Transcription/Translation and Caspase Cleavage Reactions
  - Cell Death Assay
  - Immunoprecipitation (IP) and caspase-9 assay
  - Mitochondrial potential assay
  - Conditional medium production
- QUANTIFICATION AND STATISTICAL ANALYSIS

## 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>.

## ACKNOWLEDGMENTS

We wish to thank P. Dubreuil and J.Y. Blay for c-Kit-related materials. This work was supported by institutional grants from CNRS, University of Lyon, Centre Léon Bérard and from the Ligue Contre le Cancer, INCA, ANR, ERC, and Fondation Bettencourt. H.W. and Y.S. were supported by a fellowship from the China Scholarship Council.

## 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 siCtrl 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.

Received: August 23, 2017

Revised: April 20, 2018

Accepted: August 23, 2018

Published: October 4, 2018

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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# MAB332; RRID: AB_2131313
Rabbit polyclonal anti-c-Kit	Enzo Life Science	Cat#ADI-KAP-TK005-E; RRID: AB_2038961
Mouse monoclonal anti-c-Kit-PE	Miltenyi Biotec	Cat#130-099-672; RRID: AB_2660110
Goat polyclonal anti-SCF	R&D	Cat#AF-255-NA; RRID: AB_2131621
Mouse monoclonal anti- $\beta$ -actin (C4), HRP	Santa Cruz Biotechnology	Cat#sc-47778 HRP; RRID: AB_2714189
Rabbit polyclonal anti-HA	Sigma-Aldrich	Cat#H6908; RRID: AB_260070
Rabbit polyclonal anti-caspase-3	Cell Signaling	Cat#9662; RRID: AB_331439
Mouse monoclonal anti-phospho-tyrosine	Santa Cruz Biotechnology	Cat#sc-508; RRID: AB_628122
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; RRID: AB_1120024
Rabbit polyclonal anti-Ku80	Cell Signaling	Cat#2753; RRID: AB_2257526
<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 RNAi 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	Cat# CCL-185; RRID: CVCL_0023
Human: HCT116	ATCC	Cat# CCL-247; RRID: CVCL_0291

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: IMR32	ATCC	Cat# CCL-127; RRID: CVCL_0346
Human: HMCB	ATCC	Cat# CRL-9607; RRID: CVCL_3317
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 Maise, INRA UMR754, Lyon, France	N/A
plentiCRISPR v2 vector	Dr. Carine Maise, 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, 1 mg 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 SfiI 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 rTA 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 rTA 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 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 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 1hour 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. Further, 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 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.

### 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.