



# Le récepteur à dépendance TRKC : mécanismes moléculaires et implications dans la tumorigénèse

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**The dependence receptor TRKC: molecular mechanisms and  
involvement in tumorigenesis**

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## Abstract

The neurotrophin receptor TRKC was initially shown to induce apoptosis in settings of lack of its ligand, NT-3. This cell death was described to be important in the regulation of neuronal survival during sympathetic nervous system formation and finally it was linked with several types of cancer.

During my thesis I focused on the molecular characterization of the signaling cascade leading to TRKC-induced apoptosis. Importantly, in order to kill, TRKC is double-cleaved by caspases in its intracellular domain releasing a pro-apoptotic fragment, named TRKC KF (Killer Fragment). Using a yeast two-hybrid screen we identified several potential interacting partners for TRKC KF. Initially, i focused on COBRA1, a cofactor of BRCA1.

I show here that COBRA1 is requisite for TRKC-induced cell death both *in vitro* and *in vivo*, on primary neurons. COBRA1 seems to stabilize and accumulate TRKC KF at the mitochondria, where TRKC KF induces the activation of BAX and therefore cytochrome *c* release. Therefore, it looks that TRKC-induced cell death is dependent on the intrinsic pathway of apoptosis.

During my thesis, I also took part in two projects characterizing the role of TRKC as a conditional tumor suppressor in neuroblastoma and colon cancer. We showed that in neuroblastoma tumors the pro-apoptotic function of TRKC is impaired due to an autocrine production loop of NT-3, which can be targeted as a therapeutic strategy. In colon cancer, we described another mechanism by which tumor cells evade TRKC-induced apoptosis, more specifically a loss of TRKC expression due to promoter hypermethylation.

Keywords: Dependence receptors, TRKC, caspase, COBRA1, mitochondria, BAX

## Resumé

Le récepteur à neurotrophine TRKC a initialement été montré comme induisant la mort cellulaire par apoptose en l'absence de son ligand, NT-3. Cette mort cellulaire a tout d'abord été décrite comme étant importante dans la régulation de la survie neuronale, pendant la formation du système nerveux sympathique. Plus tard, elle a été montrée comme étant impliquée dans différents types de cancer.

Au cours de ma thèse, je me suis concentré sur la caractérisation moléculaire de la cascade de signalisation conduisant à l'induction de l'apoptose par TRKC. Afin d'induire l'apoptose, le domaine intracellulaire de TRKC est clivé par les caspases en deux sites, ce qui entraîne la génération d'un fragment pro-apoptotique, TRKC KF (Killer Fragment). Plusieurs partenaires potentiels de TRKC KF ont été identifiés lors d'un crible double-hybride. Initialement, je me suis focalisé sur l'un d'eux, COBRA1, un cofacteur de BRCA1. Je montre ici que COBRA1 est requis pour la mort cellulaire induite par TRKC, à la fois *in vitro* et *in vivo*, dans des neurones primaires. COBRA1 semble stabiliser et accumuler TRKC KF à la mitochondrie, où TRKC KF entraîne l'activation de BAX et ensuite le relargage du cytochrome *c*. En conclusion, il semblerait que la mort cellulaire induite par TRKC est dépendante de la voie apoptotique intrinsèque.

J'ai aussi pris part à deux autres projets décrivant le rôle de TRKC comme un suppresseur de tumeur potentiel dans le neuroblastome et le cancer colorectal. Nous avons montré dans le neuroblastome que la fonction pro-apoptotique de TRKC est invalidée par une boucle autocrine de production de NT3, qui peut être ciblée lors d'une approche thérapeutique. Dans le cancer colorectal, nous avons décrit un second mécanisme par lequel les cellules tumorales échappent à l'apoptose induite par TrkC. Il s'agit d'une perte de l'expression de TrkC due à une hyperméthylation du promoteur.

Mots-cles: Récepteurs à dépendance, TrkC, caspase, Cobra1, mitochondrie, Bax

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## Abbreviation list

AIF	apoptosis-inducing factor
APAF1	apoptotic protease-activating factor 1
APC	adenomatosis polyposis coli
APC/C	anaphase-promoting complex/cyclosome
ASO	antisense oligonucleotides
ATP	adenosine-triphosphate
BAD	BCL-2-associated agonist of cell death
BAF180	BRG1-associated factor 180
BAK	BCL-2-antagonist/killer
BAX	BCL-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BDNF	brain-derived neurotrophic factor
BH3	BCL-2 homology domain 3
BID	BH3-interacting domain death agonist
BiFC	bimolecular fluorescence complementation
BIM	BCL-2-interacting mediator of cell death
BRCA1	breast cancer 1
CARD	caspase activation and recruitment domain
CDKN2A	cyclin-dependent kinase inhibitor 2A
C-FLIP	cellular FLICE-like inhibitory protein
cIAP	cellular Inhibitor of Apoptosis
CLL	chronic lymphoid leukemia
CNS	central nervous system
COBRA1	cofactor of BRCA1
CoIP	co-immunoprecipitation
CREB	cAMP response element-binding protein
CUL1	cullin 1

DAG	diacylglycerol
DCC	deleted in colorectal carcinoma
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferases
DP5	death protein 5
DR	dependence receptor
DRAL	down-regulated in rhabdomyosarcoma LIM-domain protein
DRGs	dorsal root ganglia
DRP1	dynamain related protein 1
EMT	epithelial-mesenchymal transition
EndoG	endonuclease G
ER	endoplasmic reticulum
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
ERK	extracellular-signal-regulated kinase
FADD	FAS-associated protein with a Death Domain
FASL	FAS ligand
FASN	fatty acid synthase
FIS1	fission 1
FOXO3a	forkhead box O3A
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GSK-3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HDAC	histone deacetylase
HEK	human embryonic kidney
Hey1	hairy/enhancer-of-split related with YRPW motif 1
HeyL	hairy/enhancer-of-split related with YRPW motif-like
HHV-8	human herpes virus 8
HRK	harakiri
HSF1	heat shock factor 1
HTRA2	high temperature requirement protein A2
ICD	intracellular domain
IMS	mitochondrial inter-membrane space
IRS1	insulin receptor substrate 1
JNK	c-jun N-terminal kinase
KF	killer fragment
KO	knock out
LOH	loss of heterozygosity
MAGI-1	membrane associated guanylate kinase domain containing 1
MAPK	mitogen-activated protein kinase
MCL-1	myeloid cell leukemia sequence 1
MFN1	mitofusin 1
MGMT	O-6-methylguanine-DNA methyltransferase

MLK	mixed-lineage kinase
MOMP	mitochondrial outer membrane permeabilization
NALP1	NLR family, pyrin domain containing 1
NF- $\kappa$ B	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGF	nerve growth factor
NLS	nuclear localization signal
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OPA1	optic atrophy 1
PA-GFP	photoactivateable GFP
PBRM1	polybromo 1
PEG	polyethylene glycerol
PI3K	phosphatidylinositol 3-kinase
PIDD	p53-induced death domain protein
PKC	protein kinase C
PLAD	pre-ligand assembly domain
PNS	peripheral nervous system
PTPC	the permeability transition pore complex
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RASSF2A	Ras association (RalGDS/AF-6) domain family member 2
RB	retinoblastoma protein
RIPK1	receptor-interacting protein kinase 1
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SCLC	small cell lung cancer
SKP1	S-phase kinase-associated protein 1
SNAI1	snail homolog 1
tBID	truncated BID
TGF- $\beta$	transforming growth factor- $\beta$
TNF	tumor necrosis factor
TNF $\alpha$	tumor necrosis factor $\alpha$
TRAIL	TNF-related apoptosis inducing ligand
TRAILR	TRAIL receptor
Trk	tropomyosin receptor kinase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
XIAP	X-linked inhibitor of apoptosis
ZEB-2	zinc finger E-box binding homeobox 2





# **1. Literature review - The neurotrophins and their receptors**

## **1.1 General considerations**

The nervous system is so highly organized and perfectly adapted to complex functions that it would have been impossible to be the result of random assembly of its simplest components. And it is not the case. The life of neurons, their differentiation, axon guidance, function and eventually death is carefully planned and modeled by trophic factors and guidance molecules, among which the neurotrophins play a key role.

The neurotrophins are a highly conserved family of secreted proteins that orchestrate neurons survival, differentiation and axon wiring towards target territories (Arevalo and Wu 2006; Huang and Reichardt 2003). The neurotrophins, which now include the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are secreted at large distances by the “target” territories (neurons in the nervous system and non-neuronal cells in the periphery) and through the establishment of a ligand gradient, act on specific populations of neurons, modulating their migratory and survival behavior. The neurons that “connect” to this neurotrophin flow will survive the period of neuronal cell death, while those that fail will eventually die.

This effect was the starting point of an amazing adventure more than 50 years ago when Viktor Hamburger, Stanley Cohen and Rita Levi-Montalcini discovered the first neurotrophin, NGF, as being essential for neuronal survival and axonal growth (Levi-Montalcini and Angeletti 1963). They showed that neurons survival was affected by modifications in target tissue size (Hamburger 1993). This was the first evidence that neuron numbers are controlled by the availability of their innervating targets. This work was awarded the Nobel Prize in 1986 and set the foundation of the “Neurotrophic Theory”: a neuron is committed to die “by

default” unless it reaches its target and receives a pro-survival signal induced by the adequate neurotrophic factor. The limited amount of trophic factor produced by the target finely tunes neurons quantity and guidance.

The neurotrophins are initially synthesized as precursors named pro-neurotrophins that are then cleaved by furin or pro-convertases to give rise to the carboxy-terminal mature neurotrophins of about 12 kDa in size (Mowla, Farhadi et al. 2001). The resulting amino-terminal residue is believed to stabilize and ensure the proper folding of the mature neurotrophins that are functionally active as stable, non-covalent dimers (Chao 2003). The neurotrophins share a highly homologous structure with well-characterized growth factors, including the transforming growth factor- $\beta$  (TGF-  $\beta$ ) and the platelet-derived growth factor. Several other neurotrophins, such as NT-6 and NT-7, were recently isolated from fish; however it seems that they do not have orthologues in mammals (Reichardt 2006).

The neurotrophins bind and activate two different classes of cell surface receptors, the TRK (A, B and C) tyrosine kinase receptors and the neurotrophin receptor p75<sup>NTR</sup>, a member of the tumor necrosis factor (TNF) receptor superfamily (Huang and Reichardt 2003). The structures of TRK receptors have in common several immunoglobulin-C2 domains and repeats rich in leucine and cysteine residues in the extracellular part while the intracellular tail contains the tyrosine kinase domain. The p75<sup>NTR</sup> receptor has four cysteine-rich extracellular repeats and lacks any kinase domain (Yano and Chao 2000).

While p75<sup>NTR</sup> is able to bind all mature neurotrophins, TRK family members exhibit ligand selectivity: NGF is the preferential ligand for TRKA, BDNF for TRKB and NT-3 for TRKC. Moreover, p75<sup>NTR</sup> acts in specific circumstances as a co-receptor for Trk receptors and its expression increases the affinity of neurotrophins for their respective receptors (Chao 2003).

## 1.2 Neurotrophin-regulated signaling pathways

The vast majority of the scientific reviews dedicated to neurotrophins are focused on the positive, pro-survival cues initiated by the neurotrophins and their receptors. By activating the TRKs tyrosine kinase receptors, neurotrophins regulate diverse pathways including the proliferation and survival of neurons, axonal and dendritic growth, cytoskeleton remodeling, synapse formation and function. In addition, the neurotrophins also have various roles outside the nervous system, namely in cardiac development, neovascularization and immune system homeostasis (Reichardt 2006).

Neurotrophin binding to TRK receptor results in receptor dimerization and autophosphorylation of several tyrosine residues by each receptor's tyrosine kinase domain. When phosphorylated, these residues become binding sites for scaffolds and enzymes that are intracellular mediators of the neurotrophin signaling cascade. In vertebrates, TRK receptors contain 10 tyrosine residues in their intracellular region, three of which (Y670, Y674 and Y675 for human TRKA) control the tyrosine kinase activity, while Y490 and Y785 serve as docking sites for different adaptors.

The major pathways engaged by neurotrophins are Ras-MAP-kinase (MAPK), PI3-kinase (PI3K), PLC- $\gamma$ 1 and their downstream effectors (Figure1).

First, activation of the small GTPase Ras by neurotrophins has been linked with neuronal survival and differentiation. The first evidence of Ras involvement in neurotrophin signaling comes from microinjection of Ras blocking antibodies in PC12 cells, which was followed by inhibited neurite formation in response to NGF treatment (Hagag, Halegoua et al. 1986). The same effect was mimicked by a mutant form of Ras (Szeberenyi, Cai et al. 1990). Transient activation of Ras is initiated by phosphor-Y490 on TRK receptors which recruits different

protein adaptors such as SHC, SH2-B, FRS2 and IRS1. In turn, activated Ras stimulates signaling through PI3- kinase, p38MAPK and c-Raf-ERK (Reichardt 2006). Both MAPK and ERK lead to activation of CREB, which in turn controls the expression of genes essential for neuron survival and differentiation (Arevalo and Wu 2006).

Secondly, PI3K-AKT is required for neurotrophins pro-survival function. More specifically, AKT phosphorylates BAD, preventing its pro-apoptotic effect (Datta, Dudek et al. 1997) and in a similar manner inhibits FKHRL1, a forkhead transcription factor for several pro-apoptotic proteins (Zheng, Kar et al. 2002). Moreover, AKT inactivates I $\kappa$ B, an inhibitor of the pro-survival NF- $\kappa$ B (Arevalo and Wu 2006).

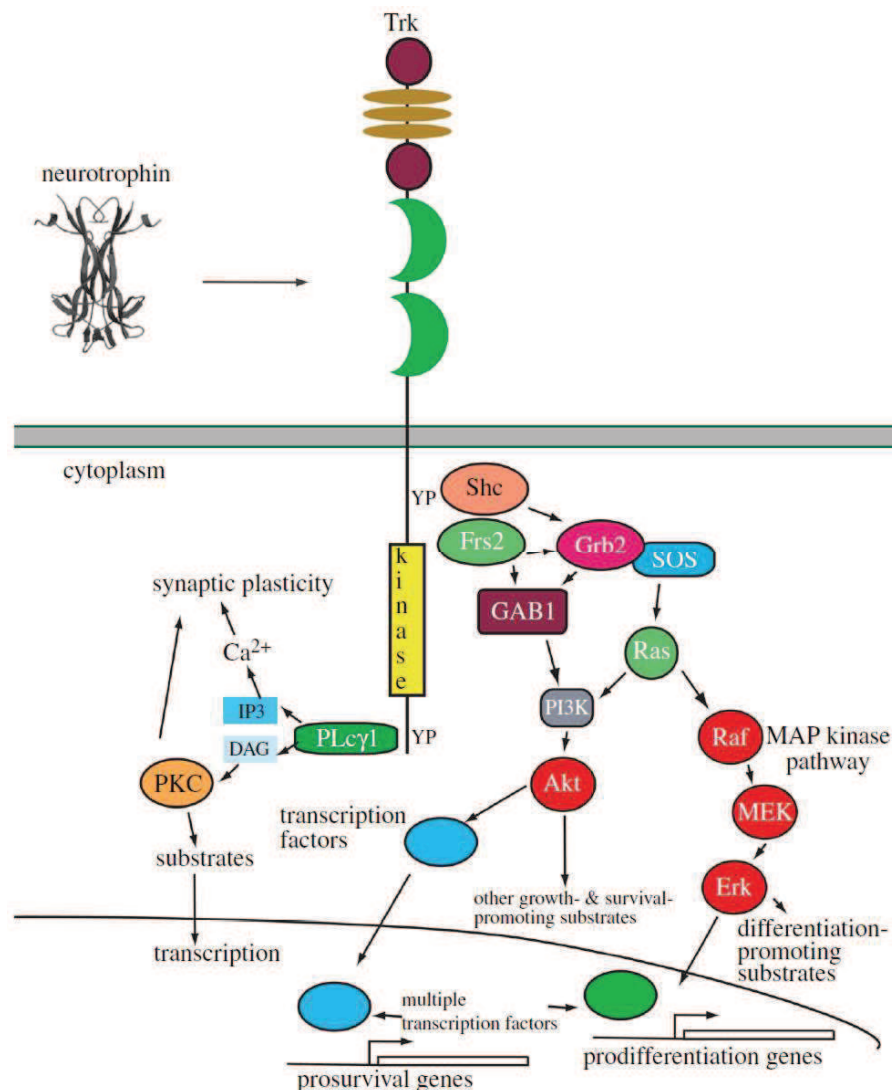


Figure 1. The neurotrophins signaling network. Adapted from Reichardt 2006.

Third, the PLC- $\gamma$ 1 pathway regulates the intracellular  $\text{Ca}^{2+}$  levels and protein kinase C (PKC) activity through generation of inositol tris-phosphate (IP3) and diacylglycerol (DAG). PLC- $\gamma$ 1 signaling seems to be important for synaptic plasticity (Bibel and Barde 2000).

### **1.3 TRKC in the development of the nervous system**

In our team we focus on the neurotrophin receptor TRKC, mixing nervous system developmental aspects with mechanistic deciphering of the pro-apoptotic function and pure cancer research. We are also investigating the link between the receptor and its ligand and several cancers like breast, colon and kidney cancer.

TRKC is located on the chromosome 15q24-q25 (McGregor, Baylin et al. 1994) and the protein product is a 145 kDa glycoprotein having a high degree of homology with both TRKA and TRKB. More precisely, there is an 85% homology for the kinase domain and only 50% for the extracellular domain (Lamballe, Klein et al. 1991). Similar to the other neurotrophin receptors, TRKC is characterized by cysteine and leucine rich domains in the extracellular part and a kinase domain in the C-terminus.

#### **1.3.1 The expression pattern of TRKC and NT-3**

In mouse, the expression of TRKC is detected very early in development, at the stage of E8 (Tessarollo, Tsoulfas et al. 1993). Later, at E9.5 the expression is associated with the neural crest cells and progressively TRKC becomes detectable in all central nervous system (CNS) and more specifically in the peripheral nervous system (PNS). In PNS, TRKC is mainly expressed in the dorsal root ganglia (DRGs). Even though TRKC is more often co-expressed with TRKA and TRKB, there are regions where only TRKC is detectable, such as the enteric nervous system (Chalazonitis 2004).

In adult, TRKC is abundantly expressed in the CNS: the cerebral cortex, the caudate putamen, the thalamus, the hippocampus and the cerebellum (Tessarollo, Tsoulfas et al. 1993). Intriguingly, TRKC-encoding transcripts are abundant on sites that are not necessarily derived from the neural crest cells. They include the heart, the lungs, submandibular glands, brown adipose tissue or the adrenal glands (Tessarollo, Tsoulfas et al. 1993; Donovan, Miranda et al. 1995).

The ligand NT-3 is expressed just in front of the migrating neural crest cells that will colonize the DRGs and also in the target territories of the sensory neurons that will then innervate the skin and the limb muscles (Farinas, Yoshida et al. 1996). This expression pattern of both TRKC and NT-3 already suggests a strong effect of TRKC/NT-3 in the spatial and temporal organization of the sensory neurons.

### **1.3.2 The role of TRKC in the DRG formation**

Gene invalidation in mice strengthened the fact that TRKC/NT-3 is important for the development of the sensory nervous system, more specifically of the DRGs. However, it was reported that other neuron populations, such as the enteric neurons, are absent in NT-3 knock-out mice (Chalazonitis, Pham et al. 2001).

The neurons constituting the DRGs are derived from the neural crest cells that are located dorsally to the neural tube and delaminate ventrally in the mesenchyme between E8 and E10. The DRGs are heterogeneous structures, being constituted of neurons very different in shape and in function. The different types of DRG neurons are specialized for the perception of a certain stimulus. Such types include the small diameter neurons expressing TRKA which mediate pain sensation (nociceptive neurons), intermediate diameter mechanoreceptive neurons expressing TRKB and large proprioceptive neurons that sense limb movements and

position. The latest express the NT-3 receptor TRKC (for review see Marmigere and Ernfors 2007).

The vast majority of neurons constituting the DRGs are generated between E10 and E13, a critical time window in which processes such as proliferation, differentiation, neurogenesis and developmental apoptosis of excess neurons, tightly coexist. At E10, TRKC is expressed in the precursors constituting the DRGs, however at E13, takes place the so-called “neurotrophin switch”: TRKC is then expressed in only 30% of the neurons, the proprioceptive ones, while the rest of the 70% express either TRKA or TRKB and are responsive to NGF and BDNF, respectively (Tessarollo, Tsoulfas et al. 1993; Farinas, Wilkinson et al. 1998).

The genetic invalidation of each neurotrophin and its receptor in mouse models has demonstrated the importance of neurotrophin signaling for the survival and differentiation of neurons in DRG. The mice invalidated for TRKA or NGF present the same number of nociceptive neurons lost at birth (Crowley, Spencer et al. 1994), the same effect being seen for the mechanoreceptive neurons in the case of the double-knock-out TRKB/BDNF (Ernfors, Kucera et al. 1995). Interestingly, the loss of NT-3 generates a loss of 70% of DRG neurons loss at birth while TRKC knock-out is accompanied by a loss of only 30% (Tessarollo, Tsoulfas et al. 1997). This suggests either a pro-apoptotic function of the receptor TRKC in settings of lack of NT-3, or another receptor able to be activated by NT-3. However this will be broadly discussed later.

It is worth noticing that TRKC signaling is not restricted to the formation of DRGs. The couple TRKC/NT-3 was also associated with the development of cochlea innervation (55-70% neurons loss in mice invalidated for TRKC) (Fritsch, Barbacid et al. 1998) and mesencephalic trigeminal nucleus (50% neuronal loss in TRKC<sup>-/-</sup> mice) (Matsuo, Ichikawa et al. 2000).



## 1.4 Neurotrophins and cell death

The cell death affecting neurons can be classified into *morphogenetic* apoptosis, leading to the formation of different neuronal populations and secondly the *pathological* apoptosis that is the consequence of injury such as cerebral ischemia and trauma, or chronic neurodegenerative diseases, like amyotrophic lateral sclerosis or Alzheimer's disease. The morphogenetic apoptosis affects between fifty and eighty percents of the neurons that are overly produced or that are unable to correctly innervate their targets (Oppenheim 1991). In spite of their different outcome in the shaping of nervous system, both morphogenetic and pathological apoptosis require similar partners and signal-transduction pathways.

Even though most of the studies focus on a pro-survival role of neurotrophins, I will focus from now on a discreet side of neurotrophins and their receptor, namely: (i) the apoptosis that can arise in the absence of neurotrophic support, (ii) in the presence of neurotrophins *via* p75<sup>NTR</sup> or (iii) the cell death triggered by unliganded TRK receptors as it is the case of the so-called "dependence receptors" (TRKA and TRKC). It would be also interesting to understand how the well-known actors of apoptosis are involved in this neurotrophin-driven neuronal cell death.

The naturally occurring cell death that was observed more than a century ago was considered for decades a passive phenomenon that was inevitable for aging cells. This view began to be shaken by studies showing that RNA and protein synthesis inhibitors delay this cell death, thus showing that active intracellular processes are required for cells to die (Martin, Schmidt et al. 1988). Another turning-point comes from the ultrastructural study of Kerr, Wyllie and Currie describing for the first time the morphological changes in dying cells (Kerr, Wyllie et al. 1972). In contrast with necrosis, in which, following injury, the cell ruptures, cells

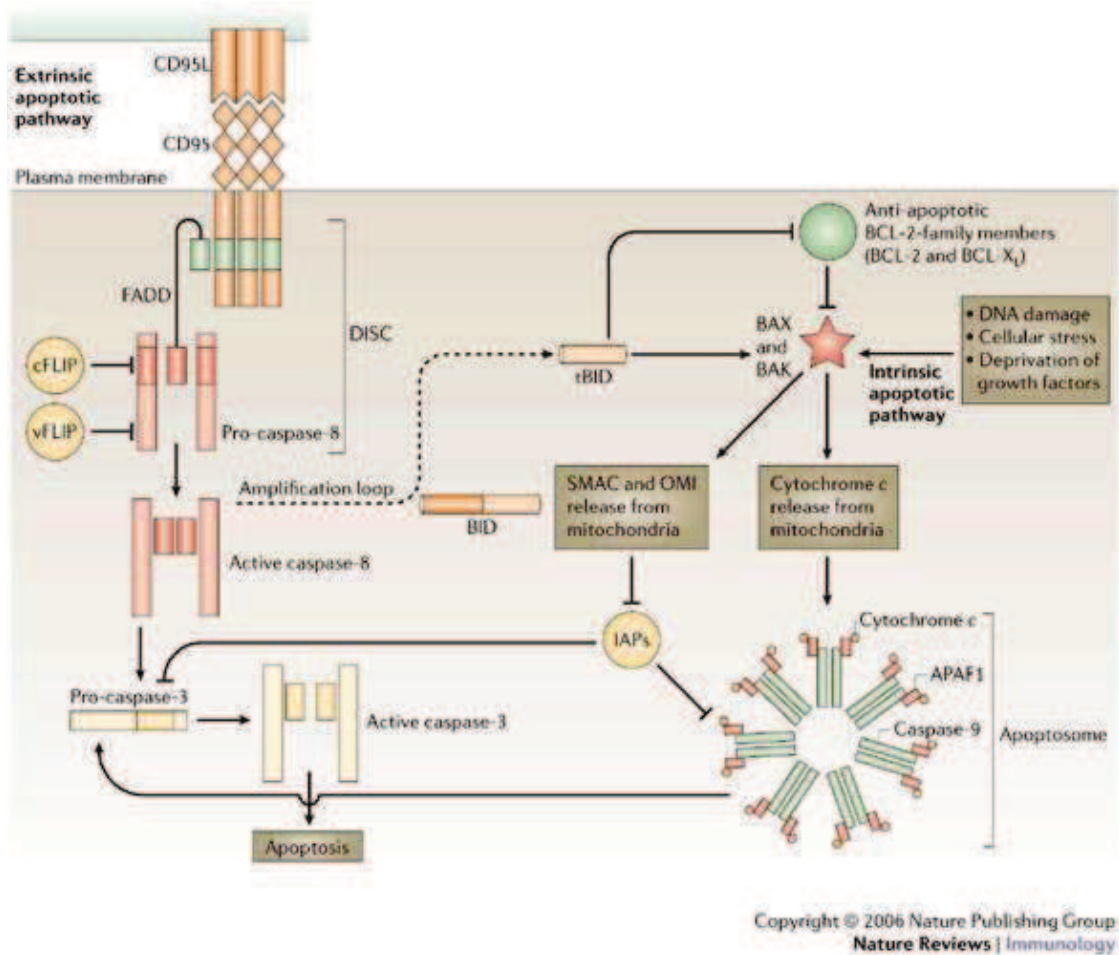
undergoing developmental death start to shrink, have condensed nuclei, retain membrane integrity and are eventually eliminated by phagocytosis. This process is called “apoptosis”.

The deciphering of apoptotic signaling cascade was given a huge leap from the genetic studies on the nematode *C. elegans*. Mutations affecting the programmed cell death allowed the identification of a core cell death machinery that is well conserved along animal species and centers around caspases, as key executioners of apoptosis (Ellis and Horvitz 1986).

#### **1.4.1 The extrinsic apoptosis**

The term of “extrinsic pathway of apoptosis” was generally used to describe a certain type of cell death initiated by extracellular signals and transduced inside the cell by specific receptors. Such death-inducing signals include the FAS/CD95 ligand (FASL/CD95L), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) or the TNF-related apoptosis inducing ligand (TRAIL). These ligands bind to specific death receptors such as FAS/CD95, TNF $\alpha$  receptor 1 (TNFR1) or TRAIL receptor (TRAILR) 1-2 (Siegel 2006; Thorburn 2004; Curtin and Cotter 2003) (Figure 2). For a better description of the extrinsic apoptosis, the example of FASL/FAS will be further detailed. In the absence of FASL, the receptor FAS is presented at the plasma membrane as trimers, stabilized by the pre-ligand assembly domain (PLAD) (Siegel, Frederiksen et al. 2000). The binding of FASL, stabilizes the trimers and induces in their intracellular domain certain conformational changes allowing the recruitment of a dynamic multiprotein complex called the DISC (Death-Inducing Signaling Complex) (Schulze-Osthoff, Ferrari et al. 1998). The DISC recruited by FAS is constituted of FADD (FAS-associated protein with a Death Domain), several C-FLIP isoforms and finally pro-caspase 8 or 10. Therefore, DISC serves as an activating platform for caspase-8 and 10 (reviewed in Schneider and Tschopp 2000).

In type I cells, for example lymphocytes, the active caspase-8 directly catalyses the activation through proteolytic cleavage of the executioner caspase-3 that will further kill the cell in a mitochondrion-independent manner. On the other hand, in type II cells (such as pancreatic  $\beta$



**Figure 2. The extrinsic and intrinsic (mitochondrial) pathways of apoptosis. From Siegel 2006.**

cells and hepatocytes) the active caspase-8 cleaves BID (BH3-Interacting Domain death agonist) leading to the generation of tBID (truncated BID), a well-known mitochondrion-permeabilizing fragment (Galluzzi, Vitale et al., 2012). Thus, type I cells do not require the mitochondria in order to finalize the intrinsic pathway, while type II cells show signs of MOMP (Mitochondrial Outer Membrane Permeabilization), including the loss of mitochondrial transmembrane potential and the release of toxic protein from the IMS

(mitochondrial Inter-Membrane Space). Among these, cytochrome *c* will assemble together with APAF1 and pro-caspase-9 and will form a complex named “the apoptosome”.

#### **1.4.2 The intrinsic (mitochondrial) apoptosis**

The mitochondrial pathway of apoptosis can be initiated by a large variety of intracellular stress signals, such as DNA damage, accumulation of unfolded protein in the endoplasmic reticulum (ER), cytosolic  $\text{Ca}^{2+}$  overload, neurotrophin deprivation and others. In spite of so many pro-apoptotic stimuli, the common point is that they all are routed to the mitochondria in which MOMP occurs (Tait and Green, 2010). MOMP is either due to the pore-forming activity of the pro-apoptotic BAK and BAX (Antignani and Youle 2006) or can result from the so-called MPT (Mitochondrial Permeability Transition) that originates in the inner mitochondrial membrane from the opening of the permeability transition pore complex (PTPC) (Brenner and Grimm 2006). MOMP is the starting point of many lethal events: the loss of mitochondrial transmembrane potential followed by the cessation of ATP synthesis and the release from the IMS of cytochrome *c*, AIF (Apoptosis-Inducing Factor), EndoG (Endonuclease G), DIABLO/SMAC, and HTRA2 (High Temperature Requirement protein A2, also named OMI) (Kroemer, Galluzzi et al. 2007).

Following MOMP, cytochrome *c* will assemble the apoptosome as described above, which triggers the activation of caspase-9 and then caspase-3 (Tait and Green, 2010). AIF and EndoG translocate in the nucleus where they mediate DNA fragmentation (Susin, Lorenzo et al. 1999; Li, Luo et al. 2001), while SMAC/DIABLO and HTRA2 bind and inhibit the anti-apoptotic function of several IAP family members (Chai, Du et al. 2000; Yang, Church-Hajduk et al. 2003).

It is well accepted that MOMP kills the cells by rapidly activating executioner caspases (caspase-dependent cell death). However, in the absence of caspase activity (i.e. APAF1 or caspase-9 deficiency), cells can undergo caspase-independent cell death most probably caused by the toxic proteins released from the IMS (Tait and Green 2008).

It is worth noticing that there are many other types of cell death such as anoikis, autophagy, entosis, necroptosis, netosis, parthanatos or pyroptosis (Galluzzi, Vitale et al., 2012), all of them with their unique features, however I will not detail them here.

### **1.4.3 Death by neurotrophin withdrawal – the case of TRKA and NGF**

The two main apoptotic pathways, the extrinsic and the intrinsic (mitochondrial) pathways, were presented to introduce basic terms and to make a more logical transition towards the neuron cell death observed in the case of neurotrophin factors withdrawal.

Following the establishment of the neurotrophic theory by Rita Levi-Montalcini (Levi-Montalcini and Angeletti 1963), scientists tried endlessly to figure out how the sympathetic neurons die in the absence of NGF.

In one of the first experiments, Martin and colleagues used inhibitors of both transcription and protein synthesis and showed that neuronal death induced by NGF deprivation requires RNA synthesis and macromolecule assembly (Martin, Schmidt et al. 1988). This was the starting point of a race for pro-apoptotic genes that may be activated by the lack of neurotrophic factors. The initial tendency was to focus on the already identified modulators of the intrinsic apoptosis.

First, Garcia and colleagues pointed towards BCL-2, a powerful anti-apoptotic protein, as being able to rescue sympathetic neurons from NGF withdrawal-induced cell death (Garcia, Martinou et al. 1992). In living cells, BCL-2 inhibits the pro-apoptotic effectors by direct

binding and heterodimerization (Chipuk, Moldoveanu et al. 2010). BAX is one of the targets of BCL-2 that is widely expressed in the developing nervous system and which is required for neuronal apoptosis. Indeed, neurons from BAX-deficient mice are protected from apoptotic stimuli (Deckwerth, Elliott et al. 1996). In addition, NGF withdrawal is followed by the redistribution of BAX from the cytosol to the mitochondria, where it induces MOMP and subsequent cytochrome *c* release (Putcha, Deshmukh et al. 1999). The sympathetic neurons cell death can be also rescued by the microinjection of a cytochrome *c* blocking antibody (Neame, Rubin et al. 1998). The brain hyperplasia observed in mice invalidated for APAF1, caspase-3 and -9 strongly suggests that the mitochondrial apoptosis is a critical regulator of the neuronal cell death in the developing brain (Kuida, Zheng et al. 1996; Kuida, Haydar et al. 1998).

Among the apoptotic inducers, two were described as being directly induced by NGF withdrawal.

First, DP5 was indentified in a genetic screen for genes up-regulated following NGF withdrawal (Imaizumi, Tsuda et al. 1997). Almost immediately, the human ortholog of DP5, called harakiri (HRK) was identified as being able to interact with BCL-2 and BCL-XL (Freeman, Burch et al. 2004). DP5 mRNA levels were the highest 15 hours after NGF removal, a time point coinciding with the neurons' commitment point to die. Moreover, the ectopic expression of DP5 was sufficient to induce apoptosis, even in the presence of NGF, which was partially rescued by BCL-2. However, BAX seems to be required for this cell death since DP5 cannot kill BAX deficient neurons (Imaizumi, Morihara et al. 1999).

DP5 transcriptional regulation seems to involve the MLK-JNK-c-jun pathway (Towers, Gilley et al. 2009). More precisely, the lack of NGF activates the stress-responsive mixed-lineage kinase (MLK) and c-jun N-terminal kinase (JNK), leading to c-jun phosphorylation and activation; the active c-jun will further bind and increase the activity of DP5 promoter.

Secondly, BIM, which is a BH3-only protein, was found to be upregulated in apoptotic sympathetic neurons (Putchu, Moulder et al. 2001). BIM (BCL2-Interacting Mediator of cell death) interacts with the antiapoptotic proteins as well as the effectors, directly inducing BAK and BAX oligomerization and MOMP (Chipuk, Moldoveanu et al. 2010).

Regarding NGF-withdrawal-induced BIM expression, several transcriptional mechanisms have been described. As for DP5, the increase in BIM mRNA is partially dependent on JNK pathway, since a dominant negative c-jun prevents neuronal cell death (Whitfield, Neame et al. 2001). Secondly, the transcription of BIM is repressed by the PI3K-AKT pathway: in the presence of NGF, AKT phosphorylates the transcription factor FOXO3a keeping it cytoplasmic and therefore inactive; however, following NGF withdrawal, FOXO3a is derepressed and translocates to the nucleus where it activates BIM transcription (Gilley, Coffey et al. 2003).

#### **1.4.4 The cell death induced by neurotrophins – the p75<sup>NTR</sup> example**

Neurotrophins can regulate two completely opposite processes, namely survival and cell death, depending on which neurotrophin receptor is expressed, in which combination, on the status of secreted neurotrophin (proneurotrophin or mature) and lastly, on cellular type. The p75<sup>NTR</sup> neurotrophin receptor summarizes best this antinomy. The pro-survival function of p75<sup>NTR</sup> can be shortly described as follow: TRKs receptors are the signaling hub and p75<sup>NTR</sup> is modulating their ligand specificity and binding strength.

Unexpectedly, NGF or BDNF activation of p75<sup>NTR</sup> has been reported to induce neuronal cell death. It was indeed showed that p75<sup>NTR</sup> mediates together with TRKA the survival of neurons during the establishment of target innervations but it induces apoptosis in the early postnatal period (Barrett and Bartlett 1994). NGF is the pro-apoptotic ligand of p75<sup>NTR</sup> for a large variety of cells including the neurons in the isthmo-optic nucleus, the retinal ganglia

cells or the mature oligodendrocytes (von Bartheld, Kinoshita et al. 1994; Frade, Rodriguez-Tebar et al. 1996; Casaccia-Bonnel, Carter et al. 1996). *In vivo*, gene invalidation studies have demonstrated that mice deficient for  $p75^{NTR}$  exhibit decreased apoptosis in the developing spinal cord and retina (Frade, Rodriguez-Tebar et al. 1996). Likewise, BDNF invalidation is characterized by an increased number of sympathetic neurons which strengthen the evidence of a link between BDNF and  $p75^{NTR}$  in the involvement of neuronal cell death (Bamji, Majdan et al. 1998). It is interesting to point out that the neurons particularly sensitive to this cell death were the ones lacking the TRK receptors, confirming that TRK expression protects neurons from neurotrophin-induced cell death (Davey and Davies 1998).

A surprising turning point in the study of  $p75^{NTR}$  came from Barbara Hempstead and colleagues showing that proneurotrophins, the unprocessed forms of the neurotrophins, are high affinity ligands for  $p75^{NTR}$ . They also found that binding of proneurotrophins to  $p75^{NTR}$  induces cell death (Lee, Kermani et al. 2001). Furthermore, in order to induce neuronal cell death, the proneurotrophins need to bind sortilin, an adaptor receptor that specifically recognizes the pro-domains of proNGF and proBDNF. In this way, sortilin serves as a switch that enables  $p75^{NTR}$ -expressing neurons to respond to neurotrophins and to undergo apoptosis rather than survival (Teng, Felice et al. 2010).

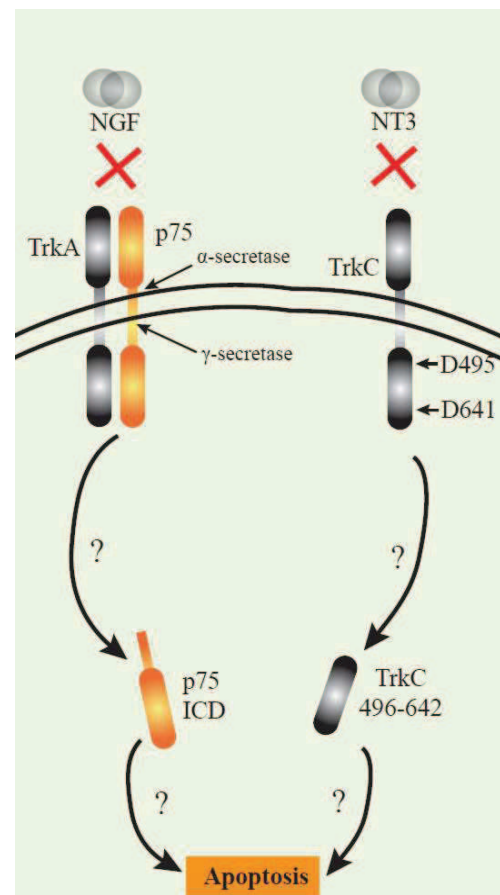
#### **1.4.5 The dependence receptors TRKC and TRKA**

The study of TRKC as a dependence receptor started following a screen for transmembrane receptors cleaved in their intracellular domain by caspases, a trait observed in most of the dependence receptors so far. It was further showed that TRKC induces apoptosis when ectopically expressed in several cell lines, the ligand NT-3 being able to rescue this cell death (Tauszig-Delamasure, Yu et al. 2007). This pro-apoptotic activity was due to a killing fragment TRKC 496-642 (TRKC KF) that was released following a double-caspase cleavage



at the aspartic residues D495 and D641 (Figure 3). The cleavage seemed to be dependent on caspase-9 and not on caspase-8 (Tauszig-Delamasure, Yu et al. 2007). Moreover, TRKC cleavage was observed *in vivo*, in sympathetic primary neurons deprived of NT-3. It is interesting to notice that an intracellular domain of TRKC mutated on D641 cleavage site acts as a dominant negative for TRKC-induced apoptosis. Preliminary data indicate this can take place by blocking the cleavage of the wild type receptor.

More importantly for the *in vivo* relevance, it was showed in collaboration with the team of Urmas Arumae in Helsinki that DRGs primary neurons cultured in the absence of NT-3 do not undergo apoptosis when microinjected with TRKC dominant negative. This experiment showed that cell death induced by NT-3 deprivation was an active process initiated by TRKC receptor itself. Furthermore, the invalidation of endogenous TRKC in these neurons and its following replacement by the un-cleavable form of TRKC protected them from NT-3 withdrawal induced apoptosis (Tauszig-Delamasure, Yu et al. 2007).



**Figure 3. TRKC and TRKA act as dependence receptors when deprived of their respective ligand. To do so, TRKC releases a pro-apoptotic fragment following caspase cleavage while TRKA seems to require the recruitment of p75<sup>NTR</sup>. From Ichim, Tauszig-Delamasure et al., 2012.**

A further validation of our studies came from the team of Yves-Alain Barde confirming that TRKC together with TRKA are dependence receptors (Nikoletopoulou, Lickert et al., 2010). In their experimental model, embryonic stem cells in which one *tau* locus was replaced with TRKA, TRKB or TRKC cDNA were differentiated into glutamatergic neurons. Most

dramatically, it was noticed that all neurons expressing TRKA and TRKC die *in vitro* in the absence of their ligand whereas TRKB-expressing neurons survive the BDNF depletion. To test whether the dependence receptor function of TRKA and TRKC can be further validated in a model of PNS development, tetraploid complementation was used to generate embryos entirely derived from the TRKA and TRKC-expressing stem cells. At E13.5 the PNS of TRKA or TRKC-expressing embryos dramatically disappears, while the embryos expressing TRKB maintain intact their nervous system.

Taken together, these results strengthen the facts that classical survival receptors such as TRKC and TRKA can behave as “dependence receptors”, by inducing apoptosis when deprived of their ligand. This opens a new perspective about the neurotrophins withdrawal-induced cell death that is not always “by default” as described above for TRKA/NGF, but rather it is an active death signaling triggered by the neurotrophin receptors.

## **1.5 TRKC and cancer**

The irony is that neurotrophin receptors were originally identified as proto-oncogenes. TRKA was first described as a fusion protein with the amino-terminal residue of a non-muscle tropomyosin. This protein product with a constitutively active kinase domain was identified in a significant fraction colon carcinoma (Martin-Zanca, Hughes et al. 1986). More recently, the fusion between ETV6 and TRKC was characterized in congenital fibrosarcoma and myeloid leukemia (Knezevich, McFadden et al. 1998) (Eguchi, Eguchi-Ishimae et al. 1999). As proto-oncogenes, the TRK receptors are being described mostly in cancers with neural and neuro-endocrine origin, however their aberrant expression was reported in other types of cancer.

### **1.5.1 TRKC/NT-3 in neuroblastoma**

Neuroblastoma, a form of cancer originating from the sympathetic nervous system, is the most common type of solid cancer in children (Henry, Tashjian et al. 2005). This form of cancer either regress spontaneously, this often happening in infants, or it progresses inexorably into multiple metastases. Neuroblastomas have been classified into 6 stages (1, 2A, 2B, 3, 4, 4S), with 1 describing localized tumors, easily operable and 4 standing for general dissemination of tumors in the whole organism (Siegel, Ishwaran et al. 2002). Currently, many genetic markers including ploidy status, allelic loss or oncogene amplification have been indentified and correlated with the clinical outcome of neuroblastoma. For instance, MYCN oncogene amplification is linked with more aggressive tumors and poor prognosis (for review Brodeur 2003).

Interestingly, the expression of TRK neurotrophin receptors is also associated with neuroblastoma outcome. Of note, high expression of TRKA correlates with good prognosis, while loss of TRKA together with MYCN amplification was described in aggressive neuroblastomas (Nakagawara, Arima et al. 1992). Human TRKA maps to 1q21, however no mutation or rearrangements have been identified to date. Furthermore, most of neuroblastomas express very low or undetectable levels of NGF. On the other hand, TRKB is highly expressed in aggressive tumors, similarly to its ligands BDNF and NT-4/5 that seems to stimulate cancer cell proliferation and metastasis (Matsumoto, Wada et al. 1995). Moreover, the up-regulation of both TRKB and BDNF in neuroblastoma is associated with resistance to drug therapy, probably by an over-activation of the PI3K/AKT survival pathway (Ho, Eggert et al. 2002).

TRKC is mainly expressed in favorable neuroblastomas. These tumors are also correlated with younger age and less aggressive stage and they lack MYCN amplification (Brodeur, Minturn et al. 2009).

In this context it is worth mentioning our recent study showing that NT-3 is up-regulated in a large fraction of aggressive stage 4 neuroblastomas tumors. This autocrine production of ligand blocks the apoptosis induced by TRKC and favors survival and dissemination of cancer cells (Bouzas-Rodriguez, Cabrera et al., 2010).

### **1.5.2 TRKC/NT-3 in medulloblastoma**

Medulloblastoma accounts for 20-25% of CNS neoplasms among children aged less than 18 years old (Pomeroy, Sutton et al. 1997). It originates in the cerebellum, it has an aggressive growth and usually metastasizes through the entire nervous system. Similar to neuroblastoma, the abundance of TRKC expression correlates with a better therapeutic response (Segal, Goumnerova et al. 1994). Interestingly, over-expression of TRKC inhibits the growth of intracerebral xenografts of a medulloblastoma cell line in nude mice (Kim, Sutton et al. 1999). Moreover, in several medulloblastomas, the ectopic expression of TRKA induces apoptosis, apparently through Ras-mediated signaling (Nakagawara 2001).

### **1.5.3 TRKC/NT-3 in other types of cancer**

The ETV6-TRKC gene fusion originating from the t(12;15)(p13;q25) translocation has been associated with congenital fibrosarcoma, acute myeloid leukemia and breast carcinoma (Lannon and Sorensen 2005) and has focused the attention of researchers on TRKC and its ligand. The expression of both TRKC and NT-3 was then assessed for several other types of cancers, such as pancreatic and prostate cancers (Ohta, Numata et al. 1997; Satoh, Mimata et al. 2001; Weeraratna, Arnold et al. 2000). Intriguingly, the malignant pancreatic and prostate

epithelial cells secrete NT-3 which will further promote their survival and proliferation. It was shown however that treatment with a NT-3 blocking antibody was able to reduce the tumor growth in a mouse model of xenografted pancreatic cancer cells (Miknyoczki, Wan et al. 2002). It is also worth noticing a study showing that TRKC is a marker of good prognosis in breast cancer (Blasco-Gutierrez, Jose-Crespo et al. 2007). Recently, we have completed a study on colon cancer, in which we have noticed a loss of TRKC in most of the tumors, due to promoter hypermethylation. More interestingly, the re-expression of TRKC was associated with tumor cell death and inhibition of tumor growth (Genevois *et al.*, submitted, PNAS).

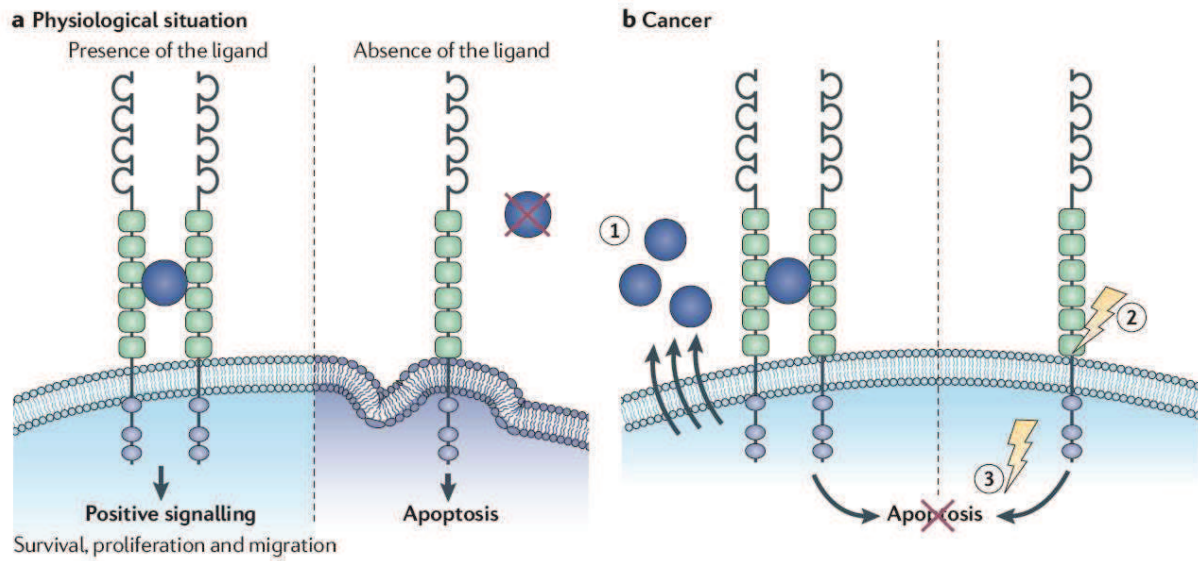
#### **1.5.4 Targeting TRKC as a therapeutic strategy**

Since the first reports showing an involvement of tyrosine kinase receptors (RTK) in cancer progression, the therapeutic industry responded with drugs designed to disrupt TRK tyrosine kinase activity. This first strategy materialized when the indolocarbazole analogues CEP-751 and its newer version CEP-701 (lestaurtinib) reached the clinical trials for treatment of various cancers such as neuroblastomas, pancreatic adenocarcinoma, medulloblastoma, prostate cancer, myelofibrosis or acute myeloid leukemia (source: <http://clinicaltrials.gov>). Lestaurtinib is a TRK-selective tyrosine kinase inhibitor that, at nanomolar concentration, blocks TRK activation by its respective ligand. Its efficacy was already proven in neuroblastoma preclinical xenograft models (Evans, Kisselbach et al. 2001). The disadvantage of these therapies is however their restricted specificity since they have side-effects caused by the inhibition of other RTKs such as the vascular endothelial growth factor receptor or the platelet-derived growth factor kinase or protein kinase C (reviewed in Brodeur, Minturn et al. 2009).

A next generation of more specific inhibitors started to be tested. Among them, AZ-23 is a potent ATP-competitive inhibitor with higher specificity for TRK receptors (A, B and C). Despite the fact that AZ-23 efficiently blocks tumor growth in murine neuroblastoma models it was later showed it has poor pharmacodynamic properties (Thress, Macintyre et al. 2009). Monoclonal antibodies against NGF and NT-3 were used in pancreatic and prostate cancer xenograft murine models with successful results (Miknyoczki, Wan et al. 2002), however the question of their specificity and side-effects remains open.

Even though TRKs inhibitors marked an important progress in cancer therapy, the tumor cells-acquired resistance is still an unsolved problem. These inhibitors are blocking the proliferation of tumor cells however, most of the time, this does not result in dramatic tumor regression since they do not induce apoptosis of malignant cells. Therefore, when drug resistance is acquired, the remaining tumor cells usually reactivate tumor growth.

Dependence receptors have been proposed as alternative therapeutic targets in various cancers, which may bring answers to the problems discussed above. In particular, the inhibition of the interaction between DCC and its ligand netrin-1 that was overly produced in breast, lung and neuroblastoma cancers (Figure 4) was shown to be effective in re-inducing apoptosis and reduce tumor growth (for review see Mehlen, Delloye-Bourgeois et al., 2011). In this context, the strategy to prevent ligand/dependence receptor binding can be extended to the TRKC/NT-3 couple that was shown to be responsible for neuroblastoma cancer progression (Bouzas-Rodriguez, Cabrera et al., 2010).



**Figure 4.** In physiological conditions, the dependence receptors induce either a positive or apoptotic signaling depending on ligand availability (a). In cancer cells (b), the dependence receptors pro-apoptotic pathway is usually disrupted following three events: an autocrine production of the ligand (1); loss of receptor function (due to LOH or promoter hypermethylation) (2); and the loss of pro-apoptotic partner(s) (3). Adapted from Mehlen, Delloye-Bourgeois et al., 2011.

## 2. Results

When I joined the “Dependence receptors, Cancer and Apoptosis” laboratory in 2009 as a PhD student, I started a project aiming at better understanding the role of TRKC as a dependence receptor and its possible therapeutic targeting. Since the article "Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TrkC-induced apoptosis” was being revised for the Journal of Clinical Investigation (JCI), I participated in this project by performing several death assays on neuroblastoma cell lines and testing different ways of inhibiting TRKC/NT-3 interaction in a murine xenograft model (siRNA and blocking antibody). This article in which I am one of the co-authors shows that TRK/NT-3 interaction can be successfully targeted in neuroblastoma resulting in tumor growth inhibition and regression.

However, my main project was to decipher the molecular mechanisms of TRKC-induced apoptosis. The starting point was a yeast two-hybrid screen using the cleavage fragment of TRKC capable of inducing apoptosis (TRKC KF) as bait. Among several interesting potential partners, I focused on COBRA1, the cofactor of BRCA1. Our experiments clearly show that COBRA1 is a pro-apoptotic partner of TRKC KF that acts as an apoptosis enhancer linking TRKC with the intrinsic (mitochondrial) pathway of apoptosis. More precisely, TRKC KF is able to partially localize at the mitochondrial membrane where it binds and activate BAX. Moreover, it is capable of releasing cytochrome *c* that will eventually form the apoptosome and activate caspase-9, leading to cell death. This work is currently under review at Science Signaling.

During my last year of thesis, I took part in a stimulating project centered on colon cancer, in which TRKC-induced apoptosis abrogated due to loss of the receptor following promoter hypermethylation. This article in which I am co-first author is under review at PNAS.



The totality of my results will be therefore presented as an overview of published and submitted articles, followed by unpublished data and ongoing projects.

## **2.1 Article 1: The dependence receptor TRKC triggers apoptosis upon COBRA1 recruitment and shuttling to the mitochondria**

We describe here the pro-apoptotic mechanism of TRKC. When deprived of NT-3, TRKC is cleaved by caspases in its intracellular domain at two aspartic residues, D495 and D641, releasing a pro-apoptotic fragment TrkC 496-642 (TRKC KF). The cleavage was observed both *in vitro* and *in vivo* on primary DRG neurons. Moreover, COBRA1, an interacting partner issued from a yeast two-hybrid screen, interacts with TRKC KF acting as an apoptotic enhancer. Indeed when co-expressed with COBRA1, the cell death induced by TRKC is significantly increased while the knock-down of COBRA1 prevents this apoptosis.

Interestingly, TRKC KF is able, due to its interaction with COBRA1, to partially localize at the mitochondrial membrane where it interacts with and activates BAX. This cooperation leads ultimately to the release of cytochrome c, formation of the apoptosome, caspase-9 and -3 activation and finally apoptosis.

To conclude, we provide here evidences indicating that TRKC is the first dependence receptor described to induce apoptosis in a mitochondrial-dependent manner.

# **The dependence receptor TrkC triggers apoptosis upon Cobra1 recruitment and shuttling to the mitochondria**

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Short title: TrkC triggers apoptosis *via* Cobra1 recruitment.

## **Abstract**

The neurotrophin receptor TrkC was recently identified as a dependence receptor and as such it triggers apoptosis in absence of its ligand NT-3. The molecular mechanism for apoptotic engagement involves the double cleavage of the receptor's intracellular domain, leading to the formation of a pro-apoptotic "killer" fragment (TrkC KF). Here we show that TrkC KF interacts with Cobra-1, a putative co-factor of BRCA1, and we demonstrate that Cobra1 is required for TrkC-induced apoptosis, both in cell cultures and in primary sensory neurons. Cobra1 recruits the TrkC KF and drives TrkC KF-mediated activation of the intrinsic apoptosis pathway. We show, by gain and loss-of function experiments, that Cobra1 triggers mitochondrial TrkC KF localization and that TrkC KF directly acts at the mitochondria to promote Bax activation, cytochrome *c* release and apoptosome-dependent apoptosis. Thus, we propose that in the absence of NT-3, proteolytic cleavage of TrkC leads to the release of a pro-apoptotic killer fragment. This killer fragment interacts with Cobra1 and shuttles to the mitochondria where it induces cytochrome *c* release and subsequent apoptosis amplification.

## Introduction

The neurotrophins NGF, BDNF, NT-3, NT-4/5 and their respective receptors p75<sup>NTR</sup>, TrkA, B and C play a critical role in the developing peripheral nervous system. The neurotrophins are produced by the future targets of the sensory neurons and promote their migration, proliferation and differentiation (Bibel and Barde, 2000). Importantly, their limited amount dictates the appropriate number of neurons. The neurotrophic theory is based on the idea that sensory neurons are naturally committed to die unless a neurotrophic factor supports their survival: this death has long been thought to result from the engagement of a "default" apoptotic cell programme. However, recent studies have brought a twist into this view: TrkC and TrkA have been shown to have the intrinsic ability to actively trigger apoptosis when unbound by their respective ligands NT-3 and NGF (Ichim et al., 2012; Nikolettou et al., 2010; Tauszig-Delamasure et al., 2007).

TrkA and TrkC therefore belong to the functional family of the so-called "dependence receptors" (DRs). These receptors have the ability to induce a "positive" signalling in presence of their ligand (proliferation, differentiation, migration...), but, unlike "classical" receptors, they are not inactive in the absence of ligand and rather trigger apoptosis (Goldschneider and Mehlen, 2010). Because of their dual signalling dependent on ligand availability DRs seem to play a crucial role both in embryonic development and tumorigenesis. Indeed, DRs are hypothesized to circumvent tumor progression and metastasis by eliminating aberrant cells proliferating in settings of limited amounts of ligand (Goldschneider and Mehlen, 2010; Mehlen et al., 2011). Along

this line it was recently formally demonstrated that the prototypical dependence receptor DCC is a tumor suppressor thanks to its pro-apoptotic activity(Castets et al., 2011). As for the role of DRs during development, it has been proposed that this ligand-dependent inhibition of apoptosis is a mechanism for regulating developmental angiogenesis(Castets et al., 2009) and for fine-tuning neurons localization and migration during nervous system development (Furne et al., 2008; Mille et al., 2009; Thibert et al., 2003). Along this line, the elegant work performed by Barde and colleagues supports the view that the intrinsic pro-apoptotic activity of TrkA and TrkC is key during nervous system development(Nikoletopoulou et al., 2010).

So far, little is known in terms of the signalling that allows DRs to trigger apoptosis upon ligand limitation. Most, but not all, DRs appear to require a preliminary proteolytic cleavage in their intracellular domains, to allow interaction with proapoptotic partners. Indeed, upon cleavage, the dependence receptor Ptc triggers apoptosis initiation following recruitment of a caspase-activating complex(Mille et al., 2009), UNC5B triggers apoptosis through the recruitment and activation of the serine threonine kinase DAPK(Guenebeaud et al., 2010; Llambi et al., 2005). However, nothing is known on how tyrosine kinase dependence receptors trigger apoptosis upon proteolytic cleavage. We have previously shown that the unbound TrkC is double-cleaved by caspase-like proteases, leading to the release of the TrkC 496-641 fragment (hereafter referred to as “killer fragment” or KF), which is essential and sufficient for cell death induction. While performing a search for putative partners of TrkC KF, we identified Cobra1 as a new interacting partner for TrkC KF. Cobra1 was initially identified as a BRCA1 cofactor, but little is known either about its role or about its actual cell

localization(Sun et al., 2011; Yamaguchi et al., 1999; Ye et al., 2001). We show here that Cobra1 is localized in the cytosol and interacts with TrkC KF. We also show that Cobra1 positively regulates TrkC-induced apoptosis. Moreover, Cobra1 silencing rescues dorsal root ganglia (DRG) neuron apoptosis caused by NT-3 withdrawal. Finally, Cobra1 shuttles TrkC KF to the mitochondria, where TrkC KF triggers cytochrome *c* release and apoptosome-dependent apoptosis. Taken together, our data support the view that TrkC hijacks the canonical mitochondrial pathway to induce apoptosis through Cobra1 recruitment.

## Results

### **Cobra1 is cytosolic and interacts with the pro-apoptotic fragment TrkC KF**

We have shown previously that unliganded TrkC is cleaved by caspase at two sites within its intracellular domain leading to the release of several intracellular fragments (Tauszig-Delamasure et al., 2007). This caspase dependent cleavage can be detected both *in vitro* and *in vivo* (Tauszig-Delamasure et al., 2007). Along this line, a TrkC fragment issued from the caspase cleavage in D495 can be detected in E12 Dorsal Root Ganglia (DRG) neurons which express TrkC and are dependent on NT-3 for their survival (Farinas et al., 1994). As shown in Suppl. Fig.1A, the TrkC fragment issued from D495 cleavage is not detected in primary DRG neurons cultured in the presence of NT-3 while addition of an antibody (AF1404) targeting TrkC extracellular domain and preventing NT-3 binding to this receptor (Bouzas-Rodriguez et al., 2010) is associated with the detection of the cleavage fragment. Similarly, In HEK 293T cells forced expression of TrkC is associated with the detection of a TrkC shorter fragment that is not detected when these cells are incubated with NT-3 or when TrkC is point mutated in D495 (Supp. Fig. 1B).

The cleavage of TrkC by caspase has been shown to be a pre-requisite for apoptosis induction as mutation of the caspase sites inhibit apoptosis induced by TrkC (TrkC DM in Fig.1AB and (Tauszig-Delamasure et al., 2007)). Interestingly TrkC localization in organized lipid microdomain at the membrane appears to be important for the dependence receptor activity of TrkC as treatment with cholesterol oxidase, which



converts cholesterol to cholestenone, and therefore disorganises the composition of the lipid microdomain, decreases TrkC ability to trigger apoptosis in the absence of NT-3 (Supp. Fig.1C).

TrkC cleavage by caspases at two sites (D495 and D641) is hypothesized to lead to the generation of 3 fragments: TrkC 1-495, TrkC 496-641 and TrkC 642-825. Forced expression in various cell lines of the TrkC 496-641 fragment lying between the two cleavage sites is associated with cell death induction, while TrkC 1-495 or TrkC 642-825 had no pro-apoptotic activity (Fig.1AB, Supp. Fig.1DEF and (Tauszig-Delamasure et al., 2007)). To further support the model in which NT-3 withdrawal is associated with the cleavage of the intracellular domain of TrkC and the subsequent formation of the TrkC 496-641 pro-apoptotic fragment, and therefore named “TrkC Killer Fragment” (TrkC KF), we show here that while NT-3 blocks TrkC-induced cell death, TrkC KF is pro-apoptotic whether NT-3 is absent or present (Supp. Fig.1G).

To first investigate how the TrkC KF triggers apoptosis and because the TrkC KF includes the kinase domain of TrkC, we first monitored whether TrkC KF could be tyrosine phosphorylated. As shown in Suppl. Fig.1H, while TrkC is tyrosine phosphorylated, a phosphorylation enhanced by NT-3 presence, TrkC KF is not detected by an anti-phosphotyrosine antibody. Moreover, a TrkC kinase dead mutation D572N (McGregor et al., 1994) inserted in TrkC KF (TrkC KF KD) is triggering apoptosis to a similar extent than TrkC KF (Supp. Fig. 1I). Thus, even though TrkC KF includes the TrkC kinase domain, these data support the view that this kinase domain is not active and is not implicated in TrkC KF pro-apoptotic activity.

In a search of TrkC pro-apoptotic partners, we thus performed a yeast two-hybrid screen using TrkC KF as bait and a mouse embryonic cDNA library as prey (Fig.1C). Of the putative interacting partners identified, presented in Fig.1C, we focused on Cobra1. Cobra1 (NELF-B, BRCA1 cofactor) was originally described as one of the four components of the negative elongation factor (NELF), shown *in vitro* to control RNA polymerase II (Pol II) pausing in transcription (Amleh et al., 2009). We first confirmed the putative interaction observed in yeast by co-immunoprecipitation in HEK293T cells transiently transfected with Cobra1 and TrkC or various TrkC cleavage fragments. Immunoprecipitations were performed pulling down either TrkC fragments or Cobra1 (Fig.1D and Supp. Fig.2AB). While a TrkC mutant deleted after the first cleavage site in a.a. 495 (TrkC 1-495) failed to interact with Cobra1 (Supp. Fig. 2B), Cobra1 interacts with full-length TrkC, TrkC KF and TrkC 642-825 (Fig.1D and Supp. Fig.2A). This interaction with TrkC was specific, as the intracellular fragment of neogenin, an unrelated dependence receptor (Matsunaga et al., 2004), fails to interact with Cobra1 (Fig.1D). TrkC KF bearing a kinase dead mutation still interacts with Cobra1 (Supp. Fig.2C), thus further supporting the independence of TrkC pro-apoptotic effect and TrkC tyrosine kinase activity. No obvious structural domains have been described for Cobra1, however the immunoprecipitation of TrkC KF with deletions on both C- and N-terminus of Cobra1 suggests that Cobra1 interacts with TrkC KF through its C-terminal part (Fig.1E). This observation is in line with the sequence that came out in the two-hybrid screen (AA 18-593). The interaction between Cobra1 and the TrkC cleavage fragments was further analyzed by confocal microscopy using COS-7 cells. In agreement with the co-immunoprecipitation data, a colocalization between Cobra1-RFP and TrkC KF-GFP

was detected (Supp. Fig.2D). To explore whether this interaction could be seen with endogenous proteins in the nervous system, immunoprecipitation was performed on mice brain tissue using a TrkC antibody. Cobra1 was pulled down with the TrkC antibody (Fig.1F). Of interest, this interaction was increased by the addition of the NT-3 blocking antibody AF1404. To demonstrate that this interaction occurs in neurons, primary DRG neurons were cultured in the presence or absence of NT-3 and the interaction of TrkC with Cobra1 was investigated by immunoprecipitation or co-localization studies. As shown in Fig.1G, a Cobra1/TrkC interaction was detected by immunoprecipitation and this interaction is mainly detected upon NT-3 deprivation. Along the same line, while in the presence of NT-3, endogenous TrkC and Cobra1 only weakly co-localize, they specifically co-localize in the absence of NT-3 (Fig.1H). Taken together, these data support that Cobra1 is a novel partner of the dependence receptor TrkC.

### **Cobra1 is required for TrkC-induced apoptosis.**

In a first attempt to evaluate the role of Cobra1 in TrkC pro-apoptotic activity, TrkC and TrkC KF-induced cell death was analysed upon over-expression of Cobra1. In A549 cells, while Cobra1 expression alone has no effect on cell death and while both TrkC and TrkC KF expression trigger cell death, Cobra1 expression significantly increased both TrkC and TrkC KF-induced cell death as shown by caspase-3 and Toxilight assays (Fig.2AB). In a loss-of-function experiment, Cobra1 was silenced by a siRNA strategy in A549 cells (Supp. Fig. 2E). As shown in Figure 2C, silencing of Cobra1 abrogates TrkC KF-induced apoptosis, as measured by caspase-3 activity. Of

interest, Cobra1-induced cell death was specific: indeed, silencing of Cobra1 had no effect on death induced by another dependence receptor Patched (Fig.2C).

We have previously shown that DRG primary neurons undergo TrkC-induced apoptosis upon NT-3 withdrawal (Tauszig-Delamasure et al., 2007). We took advantage of this *ex-vivo* model to test the involvement of Cobra1 in TrkC induced cell death. DRGs were dissociated and the sensory neurons were cultured for five days in the presence of NT-3 (Fig. 2D) or as a control in the presence of NGF. The withdrawal of NT-3 (or NGF) for 3 days leads to a significant increase in neuronal cell death. We showed in a previous study that microinjection of a dominant negative mutant of TrkC(Tauszig-Delamasure et al., 2007) rescued neurons deprived of NT-3 specifically and not those dependent on NGF. Interestingly, when a Cobra1 siRNA was microinjected instead of the TrkC dominant negative mutant in neurons, a similar rescue was observed (Fig. 2E), while this siRNA had no effect on the apoptosis of NGF-deprived neurons (Fig. 2F). Similar results were obtained when the neurons were deprived of NT-3 for 7 days (Fig. 2G). Taken together, our data demonstrate that Cobra1 is a key player in TrkC-induced apoptosis.

### **TrkC KF shuttles to the mitochondria and induces cytochrome c release**

While investigating Cobra1 cellular localization, we noticed, in addition to the previously reported nuclear localization (Aiyar et al., 2004; Ye et al., 2001), that a large pool of Cobra1 is cytoplasmic and appears in a punctiform staining suggestive of mitochondrial localization. As shown in Figure 3A, Cobra1 displays a Pearson's

coefficient of 0.5 with the Mito-Tracker, supportive of a pool of Cobra1 with a mitochondrial localization. To explore whether this localization could be seen with endogenous Cobra1 in the nervous system, we performed a sub-cellular fractionation followed by Cobra1 western blot analysis from mice brain tissue treated either with NT-3 or with the TrkC blocking antibody AF1404. As shown in Figure 3B, Cobra1 is detected in the mitochondria-enriched fraction but this presence is strongly increased when the NT-3/TrkC interaction is disrupted.

Because Cobra1 is required for TrkC KF-induced apoptosis and because a fraction of Cobra1 appears to be localised to the mitochondria, we investigated whether TrkC KF induces apoptosis through the canonical mitochondria-dependent pathway. As a first approach, we analysed the ability of TrkC KF to trigger cell death in MEF (Murine Embryonic Fibroblasts) cells deficient in caspase-9, Apaf1 or Bak/Bax. As shown in Figure 3CD and Supplementary Figure 3AB, TrkC is able to trigger apoptosis in respective wild type MEFs, while the MEFs invalidated for Bax/Bak, caspase-9 and Apaf1 were insensitive to TrkC expression. To further discriminate on the respective implication of Bax or Bak, we silenced either Bax or Bak by siRNA strategies in A549 cells. While Bax silencing efficiently blocks TrkC KF-induced cell death, Bak silencing has no effect (Fig. 3 EF), thus supporting the view that Bax but not Bak is required for TrkC-induced apoptosis. Moreover, we observed that ectopic expression of TrkC KF induces Bax activation, as shown by anti-active Bax immunofluorescence (Fig. 3G and Supp. Fig.3C).

Since several proteins, such as HER2(Strohecker et al., 2008), p53(Sayan et al., 2006), the hepatitis C virus core protein(Mohd-Ismail et al., 2009), or Bid (Li et al., 1998)

were shown to be cleaved by caspases releasing cleavage fragments that were able to localize to the mitochondria and trigger apoptosis, we further investigated whether the pro-apoptotic TrkC KF co-localized with mitochondria. GFP-tagged TrkC, TrkC KF, TrkC 495-825 or Bax were transiently expressed in COS-7 cells in which the mitochondria was labelled with the Mito-Tracker. As shown in Fig.4A and as determined from the Pearson's coefficient (Fig.4B), while full length TrkC and TrkC 495-825 showed no colocalization with the mitochondria, the pro-apoptotic TrkC KF displayed a partial overlapping with the Mito-Tracker. Similar results were obtained using TrkC KF Flag construct (Supp. Fig. 3D). These observations were further supported by sub-cellular fractionation and western blot analysis of TrkC. As shown in Fig.4C, in HEK 293T cells, while TrkC 495-825 was only detected in the cytosolic fraction, TrkC KF and Bax were both detected in cytosolic and mitochondrial fractions. Thus, a pool of TrkC KF displays a mitochondrial localization. To assay whether TrkC KF and Cobra1 were integrated in the mitochondrial membrane, mitochondria from HEK 293T cells that ectopically express TrkC KF and Cobra1 were sonicated allowing the separation of mitochondrial membrane-associated (MMAP) and soluble (MSP) proteins. As shown in Figure 4DE, TrkC KF was found exclusively associated with the mitochondrial membranes while Cobra1 resides in both compartments.

In a first attempt to determine whether, similarly to tBID(Li et al., 1998), TrkC KF acts directly at the mitochondria level to trigger apoptosis, TrkC KF was expressed in COS-7 cells and cytochrome *c* localization was monitored by immunofluorescence. Confocal analysis showed that cytochrome *c* displayed a classic mitochondrial localization in COS-7 cells expressing TrkC 495-825 or TrkC 642-825 (Fig.5A). In

contrast, COS-7 cells expressing TrkC KF, similarly to cells expressing Bax, show a diffuse cytochrome *c* staining, consistent with induction of mitochondrial outer membrane permeabilization (MOMP) (Fig.5AB). In addition, the analysis of cytosolic cytochrome *c* by immunoblot showed that TrkC KF expression is associated with cytochrome *c* release in transfected A549 cells (Supp. Fig.3E). Using HeLa cells stably expressing Omi-mCherry(Tait et al., 2012), we also observed that TrkC KF, similarly to Bax, triggers the mitochondrial release of Omi (Supp. Fig.3F).

To determine whether TrkC KF was sufficient to induce MOMP in a mitochondria-enriched system, we added purified TrkC KF, TrkC IC DM (all intracellular domain of TrkC mutated in the two caspase sites) or Bax to freshly enriched mitochondria and assessed the release of cytochrome *c*. Interestingly, TrkC KF, unlike TrkC IC DM, is able to induce cytochrome *c* release in this system, similarly to Bax (Fig.5C). Together, our results indicate that TrkC KF partially translocates to the mitochondria and triggers cytochrome *c* release.

### **Cobra1 regulates the mitochondrial localization of TrkC KF**

Because Cobra1 was shown to be involved in TrkC KF-induced cell death and because Cobra1 was localized at the mitochondria, we investigated the functional role of Cobra1 in the mitochondrial localization of the pro-apoptotic TrkC KF. As a first approach, we co-expressed Cobra1 together with TrkC KF in HEK 293T cells and monitored mitochondrial localization of TrkC KF. As shown in Figure 6AB, Cobra1 clearly strengthens TrkC KF mitochondrial localization. This finding was in line with the fact that Cobra1 enhances TrkC-induced apoptosis. We then performed a loss-of-

function experiment by silencing Cobra1 in HEK293T cells through a shRNA approach (Fig.6C). Of interest, the silencing of Cobra1 in HEK 293T cells strongly prevents the accumulation of the pro-apoptotic TrkC KF at the mitochondria (Fig.6DE). This finding was further confirmed in COS-7 cells in which TrkC KF showed a lower colocalization coefficient with the mitochondria following Cobra1 silencing (Fig.6F). As a control, Bax localization was not affected by Cobra1 silencing. These data support the view that Cobra1 is required for the mitochondrial translocation of TrkC KF.

## **Discussion**

The emergence of the dependence receptor paradigm has brought a new light on apoptosis during nervous system development and in the control of tumor progression. These receptors have been proposed to play a crucial role in the control of neuronal colonization (Furne et al., 2008; Matsunaga et al., 2004; Tang et al., 2008; Thibert et al., 2003; Nikolettou et al., 2010). They have also emerged as negative regulators of tumor progression and the DRs death pathway does indeed appear to be switched off in aggressive tumors. One way to silence this death pathway is by up-regulating the receptors' ligands (Delloye-Bourgeois et al., 2009a; Delloye-Bourgeois et al., 2009b; Mehlen et al., 2011; Mehlen and Puisieux, 2006). Along this line, we observed that aggressive neuroblastoma tumors produce NT-3 in an autocrine fashion, a selective advantage that allows them to proliferate and/or metastasize in settings of ligand limitation (Bouzas-Rodriguez et al., 2010). Interfering with ligand/DR interaction could therefore represent an alternative targeted therapy against cancer, as supported by the



fact we were able to inhibit tumor growth and metastasis by interfering with TrkC/NT-3 interaction in various pre-clinical models (Bouzas-Rodriguez et al., 2010).

However, if their role in development and cancer is now better apprehended, how these receptors engage the apoptotic process remains largely unclear. Recent studies have started to decipher the mechanisms induced by some dependence receptors in the absence of their ligand. The dependence receptor Patched was shown to recruit, in the absence of its ligand, a complex formed by DRAL and a CARD domain containing protein, TUCAN, which ultimately activates caspase-9 (Mille et al., 2009). Along the same line, the dependence receptor UNC5H2 needs DAP kinase and its subsequent dephosphorylation by PP2A in order to induce apoptosis (Guenebeaud et al., 2010; Llambi et al., 2005). However, because DRs do not usually show any structural analogy, the cell death mechanisms downstream of the receptor are probably different for each DR. Of interest, most of the tyrosine kinase receptors –*i.e.*, RET, MET and TrkC- that were shown so far to display a dependence receptor mode of action, undergo two proteolytic cleavages. This double cleavage appears in each case to release a pro-apoptotic fragment (Bordeaux et al., 2000; Foveau et al., 2006; Tauszig-Delamasure et al., 2007). We describe here that, for TrkC, this pro-apoptotic fragment recruits Cobra1. We show both in non-neuronal cells and in primary neurons that Cobra1 is required for the pro-apoptotic function of TrkC. Cobra1 was initially described in a nuclear complex called NELF (the Negative Elongation Factor) which has four components, of which Cobra1 (NELF-B)(Yamaguchi et al., 1999). This complex was shown to negatively regulate transcription elongation mediated by RNA polymerase II(Sun et al., 2011; Yamaguchi et al., 1999) and is also involved together with BRCA1 in

chromatin dynamics(Ye et al., 2001). Although most of the data reported above are centred on a nuclear function of Cobra1, it is worth noticing that there are several studies indicating that Cobra1 may have a cytoplasmic localization and therefore function. Yung and colleagues have reported a cytoplasmic localization of both endogenous and ectopically-expressed Cobra1(Yung et al., 2009). Moreover, several sub-cellular localization prediction soft-wares (<http://www.bioinfo.tsinghua.edu.cn>; <http://psort.hgc.jp/form.html>) support a cytoplasmic and even mitochondrial localization for Cobra1. In addition, in *Drosophila*, Cobra1 was suggested to act as a modulator of mitochondrial biogenesis and function, even though no mitochondrial localization of Cobra1 was formally shown(Chen et al., 2008). We report here that Cobra1 partially localizes to the mitochondria and, more importantly, that it is intrinsically linked to the shuttling of TrkC KF to the mitochondria.

Of interest, the fact that Cobra1 and TrkC KF trigger mitochondria-dependent apoptosis supports the view that some DRs may use this intrinsic pathway of apoptosis. While we initially reported that DCC, the prototypical DR, induces apoptosis independently of the intrinsic pathway(Forcet et al., 2001), it may not be a general trait. We show here that TrkC-induced apoptosis requires key players of the intrinsic pathway, such as Bax, Apaf1 and caspase 9, and we show that TrkC KF efficiently triggers cytochrome *c* release (see model Fig.6G).

Of interest, other proteins cleaved by caspase were also shown to generate pro-apoptotic mitochondrial-targeted fragments, such as the tyrosine kinase receptor HER2, which, upon caspase cleavage, releases two Bad-like fragments that localize to the mitochondria and induce cytochrome *c* release (Strohecker et al., 2008). Similar studies

were done on HER4 (Naresh et al., 2006), p53 (Sayan et al., 2006), hepatitis c virus core protein (Mohd-Ismail et al., 2009), or the tight junction protein MAGI-1 (Ivanova et al., 2007). Preliminary data suggest that TrkC KF does not bind anti-apoptotic BCL-2 family proteins and therefore is not likely to induce apoptosis by inhibiting them (data not shown). Along the same line, sequence analysis suggests the presence of a BH3-like domain in TrkC KF. Yet, we failed to detect any biological effect by extensive mutation of this BH3-like domain (not shown).

One alternative could be that TrkC KF behaves as a direct activator (Llambi and Green, 2011), by directly activating Bax and triggering its oligomerization and subsequent mitochondrial outer membrane permeabilisation. This view is supported by the fact that TrkC KF induces cell death in a strict Bax-dependent manner and that overexpression of TrkC KF triggers Bax activation. How exactly TrkC KF activates Bax remains an open question since we were not able to show a direct interaction between TrkC KF and Bax. This is probably due to the fact that this putative interaction is highly dynamic and has a very low affinity. Therefore it is not detectable using classical biochemistry assays. This point of view is supported by different studies suggesting that even *bona fide* Bax activators such as Bim or Bid interact very weakly with Bax, in a type of “hit and run” manner (for review see (Walensky and Gavathiotis, 2011)).

The notion that the intrinsic apoptosis pathway is central for TrkC-induced cell death is consistent with previous observations of this pathway’s involvement in neuronal death associated with neurotrophin limitation. Indeed, it has been shown that the loss of TrkC-expressing proprioceptive neurons in NT-3 mutant mice is rescued by the deletion of Bax (Patel et al., 2003). Along the same line, the number of DRG TrkC-positive

neurons is increased in Bax-deficient one month-old mice (Suzuki et al., 2010), and decreased in Bcl-x mutants (Urase et al., 1999). Interestingly, co-invalidation of caspase 9 and Bcl-x in Bcl-x/caspase-9 double mutants totally rescues the dying neuroblasts observed in Bcl-x deficient mice (Zaidi et al., 2001). However, cell death induced in the absence of neurotrophic support has been largely considered as the consequence of a passive engagement of the intrinsic apoptotic pathway; in other words a process "by default". Here, we show that TrkC actively triggers the intrinsic apoptotic pathway upon release of TrkC KF.

The cleavage of TrkC is the initiating step for the engagement of this death program. Of interest, both TrkC and TrkA were shown to be dependence receptors and to actively trigger apoptosis in the absence of their respective ligands (Ichim et al., 2012; Nikolettou et al., 2010; Tauszig-Delamasure et al., 2007). However, while it appears that TrkC is cleaved by caspases in its intracellular domain, TrkA is not (Ichim et al., 2012; Nikolettou et al., 2010), suggesting that, while TrkC cleavage is required to release the TrkC KF, TrkA probably triggers cell death by another mechanism. Barde and colleagues have proposed that TrkA-induced cell death requires the recruitment of  $p75^{NTR}$ , which serves as the trigger for induction of cell death (Nikolettou et al., 2010). While other mechanisms are probably involved in  $p75^{NTR}$ -mediated cell death (Ichim et al., 2012),  $p75^{NTR}$  may trigger apoptosis through activation of this intrinsic pathway *via* a C-terminal domain with the intrinsic ability to induce cytochrome *c* release (Rabizadeh et al., 2000; Wang et al., 2000). Together with the view that TrkA-induced cell death is associated with cleavage of  $p75^{NTR}$  (Nikolettou et al., 2010), it is tempting to speculate that both TrkC and TrkA trigger

apoptosis *via* activation of the intrinsic pathway. Further work will have to determine the precise biochemical mechanisms allowing mitochondrial membrane permeabilisation by TrkC KF or the intracellular domain of p75<sup>NTR</sup>. It will be interesting to determine whether the cleavage of TrkC by caspases is the only mechanism used by TrkC to trigger engagement of the intrinsic apoptosis pathway, or whether p75<sup>NTR</sup>, which has been shown to interact with TrkC (Bibel et al., 1999), may complement this mechanism.

## **Materials and Methods:**

### **Cell culture and transfection**

A549, HEK 293T, MEF, COS-7, MCF10A and HeLa Omi-mCherry cells were grown in DMEM (Life Technologies), supplemented with 10% FBS (Lonza). The plasmid constructs were transfected using Calcium Phosphate or JetPrime (PolyPlus) and the siRNA was transfected using Lipofectamine RNAiMAX (Life Technologies) following manufacturer's instructions.

### **Plasmid construction**

The full length TrkC, TrkC 1-495 and Neogenin ICD GFP were described elsewhere (Tauszig-Delamasure et al., 2007) (Goldschneider et al., 2008). TrkC 496-641 Flag (TrkC KF Flag) and TrkC 642-825 Flag were subcloned into the p3XFlag-CMV10 (Sigma-Aldrich) using PCR-obtained inserts with the following primers 5'-CGCAAGCTTGCTGGGCCGGATACAG-3' containing the HindIII restriction site and 5'-CGCGGTACCAgTTACACAAGGATCATCGCATC-3' with the KpnI restriction site for TrkC KF Flag and 5'-CGCAAGCTTGGACAGCCACGCC-3' containing the HindIII restriction site and 5'-CGCGGTACCGACTAGCCAAGAATGTCC-3' with the KpnI site for TrkC 642-825 Flag construct. The TrkC KF Kinase Dead D572N (TrkC KF KD Flag) plasmid was done by site-directed mutagenesis on the TrkC KF Flag plasmid using the following primers 5'-GATGCTTGTGGCAGTGAACGCCCTGAAGGATCCC-3' and 5'-GGGATCCTTCAGGGCGTTCACTGCCACAAGCATC-3'. TrkC IC DM plasmid coding for

the intracellular part of TrkC mutated for both cleavage sites (TrkC IC D495N/D641N) was subcloned into the p3XFlag-CMV10 plasmid between KpnI and XbaI sites (Tauszig-Delamasure et al., 2007). Full length TrkC GFP, TrkC KF GFP, TrkC 495-825 GFP and TrkC 1-495 GFP were subcloned into the pEGP-N3 (Clontech) using the following primers: 5'-CGCAAGCTTCGATGGATGTCTCTCTTTGC-3' containing HindIII restriction site and 5'- CGCGGTACCGCCAAGAATGTCC-3' with KpnI restriction site for TrkC GFP; 5'- CGCGCTAGCATGGATGCTGGGC-3' with NheI restriction site and 5'- CGCAAGCTTCATCCACAAGGATCATCGCATC-3' with HindIII restriction site for TrkC KF GFP; 5'- CGCAAGCTTCGATGGCTGGGCCGG-3' with HindIII restriction site and 5'- CGCGGTACCGCCAAGAATGTCCAGG-3' with KpnI restriction site for TrkC 495-825 GFP; 5'- CGCAAGCTTCGATGGATGTCTCTCTTTGC-3' with HindIII restriction site and 5'- CGCGGTACCCAGCGATGAAGGTG-3' with KpnI restriction site for TrkC 1-495 GFP. Cobra RFP plasmid was designed by subcloning the PCR-amplified human Cobra1 with the 5'- CGCGAATTCTTTCGCGGGGCTGC-3' primer containing the EcoRI restriction site and 5'- CGCGGATCCGAGCGGGGC-3' primer with the BamHI restriction site into the pEYFP-C1 plasmid (Clontech) in which the YFP was previously replaced with RFP coding cDNA. Cobra1.1 Flag and Cobra1.2 Flag plasmids were designed through site-directed mutagenesis (Stratagene) using the following STOP codon-containing primers: 5'- GCAGTACATCCTGTAGAAGGAGAGCGC-3' and 5'- GCGCTCTCCTTCTACAGGATGTACTGC-3' for Cobra1.1 Flag and 5'- GAGCTGGTTCGGCTAGGAGACACTGCC-3' and 5'- GGCAGTGTCTCCTAGCCGACCAGCTC-3' for Cobra1.2 Flag. The Cobra1.3 Flag plasmid was obtained by subcloning into p3XFlag-CMV10 the PCR-generated insert

using 5'- CGCGAATTCAGAGACACTGCCC-3' containing EcoRI restriction site and 5'- CGC GGTACCGATCAGAGCGGGGC-3' with KpnI restriction site. Bax GPF plasmid was purchased from Addgene, the Cobra1 Flag plasmid was a gift from Pr. Rong Li (UT Health Health Science Center, San Antonio, USA) while TrkA and TrkB were kindly provided by Pr. Yves-Alain Barde (Biozentrum, Basel, Switzerland).

### **Two-Hybrid screen**

The two-hybrid screen was performed by Hybrigenics (Paris, France) using the Mouse Embryo Brain RP2 library as a prey and pB27 (N-LexA-bait-C fusion) and pB66 (N-GAL4-bait-C fusion) vectors. TrkC KF construct was used as bait.

### **Cell death assays**

Caspase-3 activity was measured as described before (Bouzas-Rodriguez et al., 2010) using the Ac-DEVD-AFC substrate assay (Biovision, K105-400) while total cell death index was measured using the ToxiLight assay from Lonza (LT07-117).

### **Immunoprecipitation and western blotting**

HEK293T cells or a fresh dissected mouse brain (C57Bl/6) were lysed in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA and 0.1 to 1% NP-40 in the presence of proteases inhibitors, and further incubated with either anti-GFP (Torrey Pines Biolabs, TP401), anti-Cobra1 (Santa Cruz, sc-79991), anti-Flag (Sigma Aldrich, F3165) or anti-HA (Sigma Aldrich, H6908) and then with protein A-Sepharose (Sigma Aldrich) to pull down the



proteins of interest. For western blotting we used the additional antibodies: anti-panTrk (Santa Cruz, sc-139), anti-TrkC (Santa Cruz, sc-117), anti-Actin (Chemicon, MAB1501R), anti-GAPDH (Santa Cruz, sc-25778), anti-COXIV (Abcam, ab14744), anti-cytochrome *c* (Abcam, ab90529) and anti-phosphotyrosine (Millipore)

### **Immunofluorescence**

COS-7 cells, A549 cells or the DRG neurons were cultured on coverslips, then fixed 20 minutes in 4% paraformaldehyde and permeabilized in PBS / 0.2% Triton. The cells were then incubated for 1 hour with the appropriate primary antibody: anti-panTrk (Santa Cruz, C14, 1/150), anti-TrkC (Santa Cruz, H300, 1/100), anti-Cobra1 (Santa Cruz, 1/150) and anti-cytochrome *c* (Pharmigen, 65971A, 1/300). After 3 washes in PBS, the cells were incubated for 1 hour with the respective secondary antibody coupled FITC or Cy3 (Jackson ImmunoResearch). The DNA was stained using To-Pro (Life Technologies) and the mitochondria were stained with Mito Tracker Red CMXRos (Life Technologies). Images were obtained by confocal microscopy and analyzed using Adobe Photoshop, ImageJ or LSM Image Browser.

### **Sub-cellular fractionation and cytochrome *c* release**

The mitochondria were obtained from HEK293T cells using the Mitochondria Isolation Kit from Thermo Scientific (89874) according to the manufacturer's instructions. The mitochondria were resuspended in Mitochondria Resuspension Buffer (200 mM sorbitol, 50 mM sucrose, 10 mM succinate, 10 mM Hepes pH 7.4, 0.1% BSA, 5 mM KPO<sub>4</sub> pH 7.4; solution at pH 7.5). The mitochondria were aliquoted and incubated at 37°C for 1h

with 50 ng of TrkC KF Flag, TrkC IC DM Flag and Bax HA that were produced by in vitro translation (IVT) in wheat germ extract (Promega, L4380) as described by the manufacturer. The cytoplasmic fraction was obtained using digitonin (Sigma Aldrich) treatment. Briefly, the cells were washed once in PBS then resuspended in a solution of 70 mM Tris and 250 mM sucrose, pH 7.0. Digitonin was added at a final concentration of 0.05 mg/ml. The cells were then immediately centrifuged at 3000 rpm for 5 min and the resulting supernatant was collected as cytosol.

### **Mitochondrial sub-fractionation**

The separation of the inter-membrane space (IMS) and matrix proteins from those that are membrane bound was done as previously described (Stojanovski et al., 2007). Briefly, the isolated mitochondria were resuspended in extraction buffer consisting of 10 mM MOPS-KOH, pH 7.2 and 250 mM NaCl and then sonicated (3 times 5 pulses, 30% duty cycle). For the separation, the extract was centrifuged at 20000 g for 1 hour at 4°C. The pellet contained membranes and membrane-associated proteins and the supernatant contains liberated soluble proteins. The supernatant was further concentrated using acetone.

### **Brains extract mitochondrial enrichment**

Mitochondria were enriched in mouse adult brain extract as previously described (Fernandez-Vizarra et al., 2011). Shortly, the brain slices were resuspended in 1 ml of AT buffer (75 mM sucrose, 225 mM sorbitol, 1 mM EGTA, 0.1% BSA and 10 mM Tris-HCl, pH 7.4) and homogenized by 40 strokes with a glass/teflon Potter homogenizer.

The homogenate was centrifuges at low speed (1000g for 5 min at 4°C) and the supernatant was spun at 9000g for 10 min at 4°C. The pellet enriched in mitochondria was washed twice in the AT buffer and further processed.

### **Sensory neuron dissociation, culture and injection**

For the immunofluorescence, DRG neurons were dissected from OF1 mice at the E14 embryonic stage, dissociated and cultured on polylysine (Sigma Aldrich) and in a cultured medium DMEM F12 (Life Technologies) supplemented with B27 (Life Technologies) and 50ng/ml NT-3 (Abcam) during 4 days before labelling. For the microinjection studies, the neurons were grown and injected as published(Tauszig-Delamasure et al., 2007), except that the culture medium was Neurobasal supplemented with B27 (Life Technologies).

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The authors declare to have no conflict of interest in this study.

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## Figure Legends

### Figure 1. Identification of Cobra1 as TrkC interacting partner.

(A,B) TrkC and TrkC 496-641 (TrkC KF) induce cell death in A549 (A) and HEK293T (B) cells as shown by caspase-3 activity and trypan blue exclusion assay, respectively. (C) The yeast two-hybrid system used for screening for TrkC KF interacting partners. (D) Immunoprecipitation of TrkC GFP, TrkC KF GFP, TrkC 642-825 GFP and Neogenin ICD GFP in HEK 293T transfected cells. All proteins were immunoprecipitated using anti-GFP antibody and Cobra1 was revealed by an anti-Flag western blot. (E) Graphical representation of the Cobra1 deletion constructs and immunoprecipitation of Cobra1.1, Cobra1.2 and Cobra1.3 using anti-Flag antibody; the interaction with TrkC KF was revealed by an anti-GFP antibody western blot. Data represent mean  $\pm$  SEM.  $*P < 0.05$ ,  $***P < 0.001$ , 2-sided Mann-Whitney test, compared with control. (F) Immunoprecipitation of TrkC in adult mouse brain lysate. Cobra1 was revealed by western blot with an anti-Cobra1 antibody. (G) E14 DRG neurons were cultured with NT-3 for 5 days and deprived of trophic factor for 12 hours and immunoprecipitated with anti-Cobra1. TrkC was revealed by western blot. (H) Immunofluorescence on E14 DRG neurons with anti-TrkC and anti-Cobra1 antibodies. Line scans demonstrating the colocalization between TrkC and Cobra1. NT-3 deprivation occurred 12 hours before the immunostaining. Scale bar represent 50  $\mu$ m. (I) Plot of Pearson's coefficient depicting the colocalization between TrkC and Cobra1 in DRG neurons.

**Figure 2. Cobra1 enhances TrkC-induced apoptosis both *in vitro* and *in vivo*.**

(**AB**) Co-expression of Cobra1 enhances cell death induced by both TrkC and TrkC KF as shown by caspase-3 in A549 cells (**A**) and by Toxilight in HEK 293T cells (**B**). (**C**) Cobra1 silencing rescues the cell death induced by TrkC KF in A549 cells as measured by the caspase-3 assay. Data represent mean  $\pm$  SEM.  $*P < 0.05$ ,  $***P < 0.001$ , 2-sided Mann-Whitney test, compared with control. (**D**) The experimental model used of the neurotrophic assay on the microinjected DRG sensory neurons. DIV: days *in vitro*. (**E, F, G**) Sensory neurons were maintained with either NT-3 or NGF, microinjected with the plasmids for scrambled or Cobra1 shRNA and cell death induced by unliganded TrkC or TrkA was quantified 3 and 7 days later. Data represent mean  $\pm$  SEM (n=4).  $***P < 0.001$ , ns (non significant) 2-sided Mann-Whitney test, compared with control.

**Figure 3. TrkC KF-induced apoptosis involves the mitochondrial pathway.**

(**A**) Endogenous Cobra1 partially localizes at the mitochondria as shown by confocal analysis in A549 cells (left panel) and by the associated Pearson's coefficient (right panel). (**B**) Cobra1 is enriched in the mitochondrial fraction in brain extract upon NT-3/TrkC interaction inhibition. Adult mouse brains were incubated for 6 hours with NT-3 (100 ng) and AF1404 (200  $\mu$ g) and the brain extract were fractioned before immunoblotting for Cobra1, Bax and COX IV. (**C,D**) Ectopic expression of TrkC and TrkC KF induces apoptosis in wild type MEFs while it has no effect on MEFs invalidated for Bax/Bak, caspase-9 and Apaf1. Briefly, MEF cells were transiently transfected with the indicated construct and cell death was measured after 24h in low serum medium using either caspase activity assay (C) or Toxilight (D). (**E**) The invalidation of Bax in

A549 cells prevents TrkC KF-induced cell death. The efficacy of the siRNA Bax was tested on HEK 293T cells in which Bax GFP was ectopically expressed. (F) Same as in (E) using siRNA for Bak. The efficacy of siRNA Bak was tested on endogenous Bak using A549 cells. (G) TrkC KF, similarly to actinomycin D, is able to activate Bax as shown on Cos7 cells immunostained with the Bax 6A7 (active Bax) antibody.

**Figure 4. TrkC KF partially localizes at the mitochondria.**

(A) Confocal analysis of COS-7 cells revealing a partial colocalization of TrkC KF GFP with the mitochondria, stained by Mito Tracker Red CMXRos. (B) Pearson's coefficient analysis of the colocalization of TrkC GFP, TrkC KF GFP, TrkC 495-825 and Bax GFP with the mitochondria. Data represent mean  $\pm$  SEM. \*\*\* $P < 0.001$ , ns (non significant) 2-sided Mann-Whitney test, compared with control. (C) HEK293T cells were transfected with the same constructs as in (A) and then fractionated into cytosolic (C) and mitochondrial fractions (M). Arrows indicate the molecular weights of the different constructs. (D,E) Mitochondria were isolated from HEK 293T cells expressing TrkC KF Flag and Cobra1 Flag and fractionated into membrane-associated (MMAF) and soluble proteins (MSP).

**Figure 5. TrkC KF induces cytochrome c release.**

(A) Confocal analysis in TrkC KF transfected COS-7 to study cytochrome c release. COS-7 cells were stained with an anti-cytochrome c antibody. Cells displaying a representative cytochrome c release in the cytoplasm are indicated by arrow heads. (B) The percentage COS-7 cells with released cytochrome c was quantified. (C)

Mitochondria from HCT116 cells were incubated at 37°C for up to 60 minutes with 50 ng of TrkC KF Flag, TrkC IC DM Flag and Bax HA that were produced by in vitro translation (IVT) in wheat germ extract. Cytochrome c is detected in the pellet (P) and in the supernatant (S) fractions.

**Figure 6. Cobra1 controls the mitochondrial localization of TrkC KF.**

**(A)** The ectopic expression of Cobra1 in HEK 293T cells enhances the mitochondrial localization of TrkC KF. Cell fractionation was performed. The mitochondrial (M) and cytosolic (C) fractions are indicated. “Short” and “Long” indicate different exposure times of the GFP immunolabeling and quantification as a ratio (M/C : mitochondrial fraction over cytosolic fraction) is shown in **(B)**. **(C)** Immunoblot showing Cobra1 level upon transfection with the shCobra1 plasmid. Cobra1 was expressed in HEK293T cells together with increasing concentration of shCobra1 plasmid. **(D)** Cobra1 silencing impairs TrkC KF mitochondrial localization. TrkC KF GFP was transfected in HEK293T cells together or not with shCobra1 and cell fractionation was performed. GFP immunoblot is shown. The intensity of the bands has been quantified and is represented as a ratio (M/C : mitochondrial fraction over cytosolic fraction) in **(E)**. **(F)** The silencing of Cobra1 in COS-7 cells reduces the colocalization index (Pearson’s coefficient) between TrkC KF GFP and the Mito Tracker. Data represent mean  $\pm$  SEM. \*\*\* $P < 0.001$ , ns (non significant) 2-sided Mann-Whitney test, compared with control. **(G)** Model of Cobra1’s involvement in TrkC-induced apoptosis *via* the mitochondrial intrinsic pathway. The unliganded TrkC is cleaved by caspases, releasing TrkC KF that localizes at the mitochondria. This shuttling seems to require Cobra1. Furthermore, TrkC KF is able to

release cytochrome *c* and thus initiates the classical cascade of the intrinsic apoptosis.

**Supplementary Figure 1. TrkC pro-apoptotic activity is described in settings of receptor cleavage, NT-3 availability, cellular localization and kinase activity**

**(A)** DRGs from E12 mouse embryos were dissociated and incubated for 12 hours with either NT-3 or AF1404. Protein extracts were immunoblotted with an anti-TrkC antibody raised against the C-terminal domain of TrkC. **(B)** The cleavage of TrkC 1-640 GFP, which conserves the first cleavage site D495, was assessed in HEK 293T cells in the presence or absence of NT-3. NT-3 treatment and D495N mutation prevent the detection of a smaller fragment. GFP immunoblot is shown. **(C)** Caspase-3 assay on A549 cells transfected with TrkC or UNC5B treated or not with cholesterol oxidase (2U/ml). **(D,E,F)** Caspase-3 and Toxilight assays on A549 cells **(D)** and Cos-7 cells **(E,F)** transfected with the indicated constructs. **(G)** Caspase-3 assay on A549 cells, transfected with either TrkC or TrkC KF and treated or not with NT-3. **(H)** Transfected TrkC KF GFP in HEK 293T cells was immunoprecipitated and probed with an anti-phosphotyrosine antibody. **(I)** Caspase-3 assay on A459 cells with the mutant TrkC KF Kinase Dead (TrkC KF KD). For caspase-3 and Toxilight assays, data represent mean  $\pm$  SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , 2-sided Mann-Whitney test, compared with control.

**Supplementary Figure 2. Cobra1 interacts with the TrkC receptor.**

**(A)** Cobra1 was immunoprecipitated in HEK293T cells using an anti-Cobra1 antibody and the interaction with TrkC KF GFP and TrkC 642-825 GFP was revealed by an anti-GFP western blot. **(B)** While Cobra1 interacts with full length TrkC, it does not with TrkC

1-495. **(C)** Immunoprecipitated TrkC KF KD GFP interacts with Cobra1. **(D)** Confocal imaging on COS-7 cells showing the colocalization between Cobra1 RFP, TrkC KF GFP and TrkC 1-495 GFP. **(E)** The efficacy of Cobra1 siRNA on ectopically expressed Cobra1 in HEK 293T cells (left panel) and endogenous Cobra1 in A549 cells (right panel). A scramble siRNA (siRNA scr) is used as a control.

**Supplementary Figure 3. TrkC KF causes the release of the mitochondrial cytochrome *c* and Omi.**

**(A,B)** Trypan blue cell death assay on wild type **(A)** and Bak/Bax-deficient **(B)** MEFs. **(C)** Cos7 cells were transfected with TrkC KF GFP and TrkC 496-825 GFP and immunostained for active Bax (6A7 clone). Actinomycin D (500 ng/ml) was used as positive control. **(D)** MCF10A cells were immunostained with anti-Flag antibody following transfection of TrkC KF Flag. Mito Tracker Red CMXRos was used to stain the mitochondria. **(E)** TrkC KF induces the release of cytochrome *c* from the intermembrane mitochondrial space as revealed by the anti-cytochrome *c* western blot on the cytoplasmic fraction of A549 cells obtained by digitonin permeabilization. **(F)** Confocal imaging on Hela cells stably expressing Omi-mCherry showing the mitochondrial release of Omi when TrkC GFP, TrkC KF GFP or Bax GFP are ectopically expressed but not when TrkC 495-825 GFP is expressed. Arrow heads show the diffuse labelling of Omi when it is released in the cytoplasm.



Figure 1

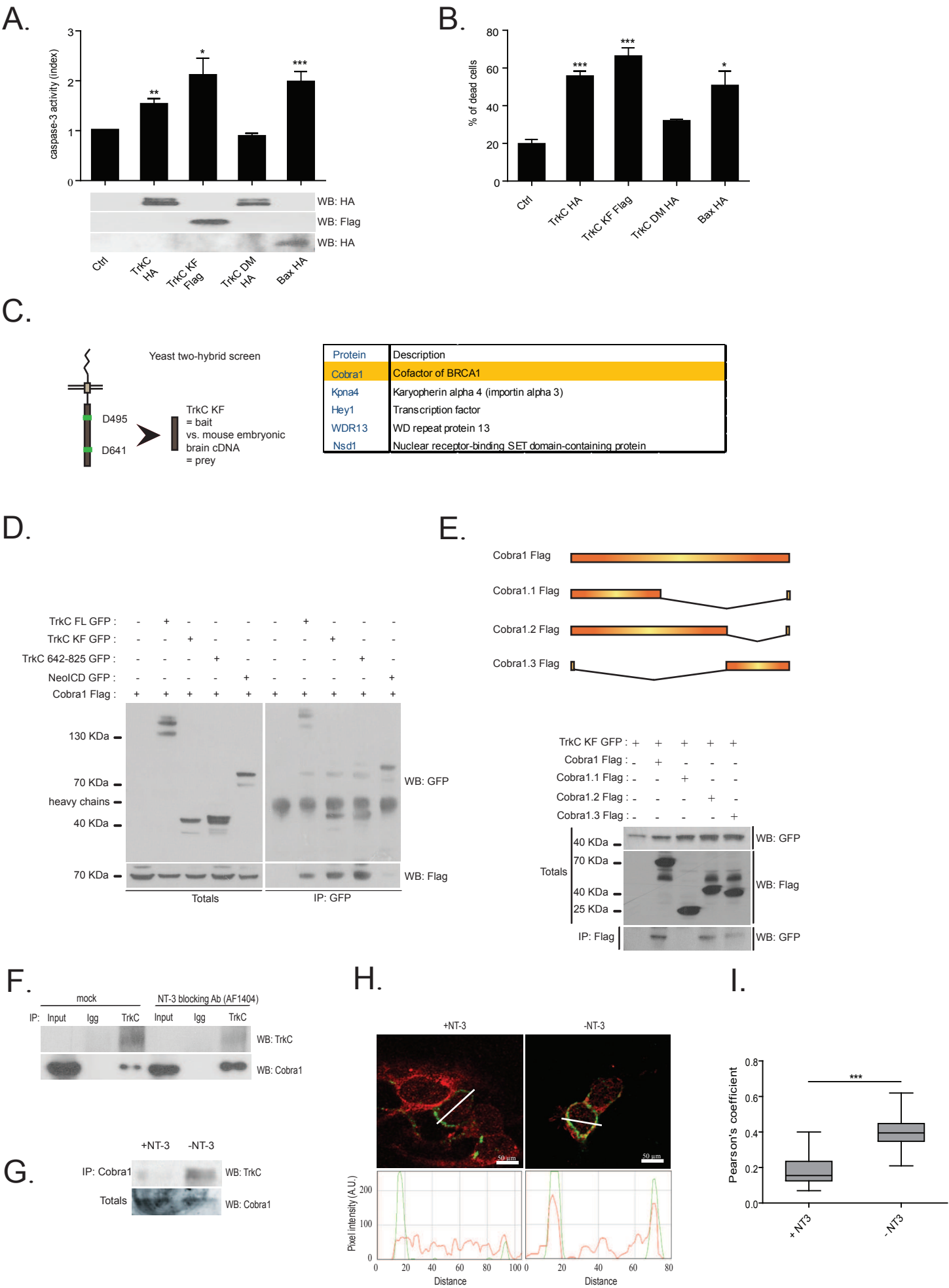


Figure 2

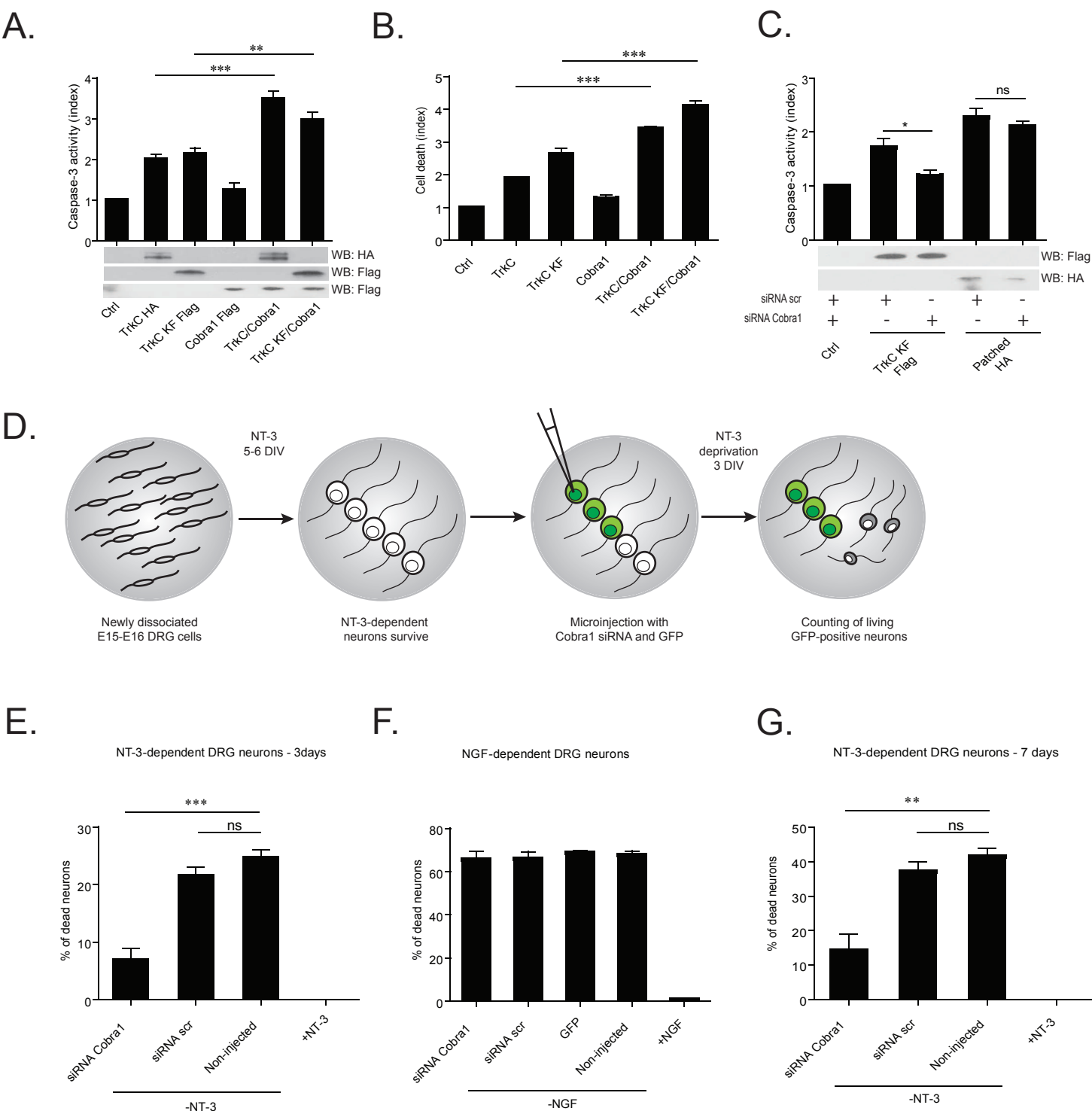
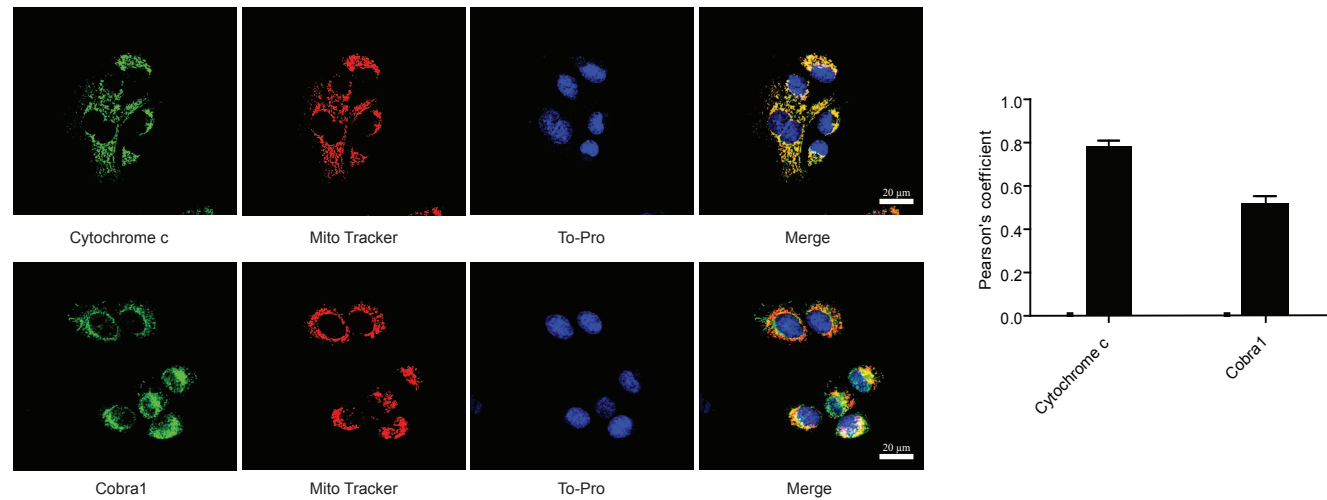
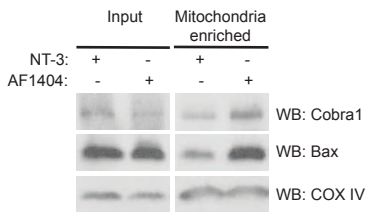


Figure 3

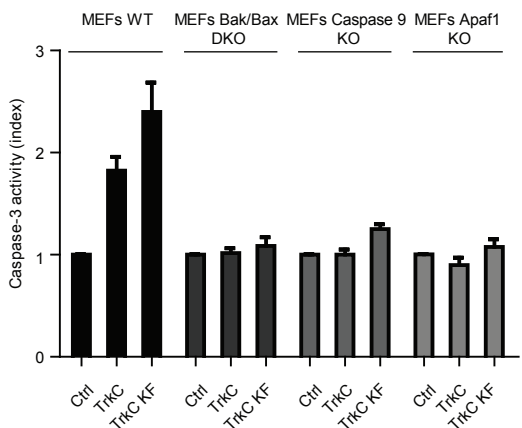
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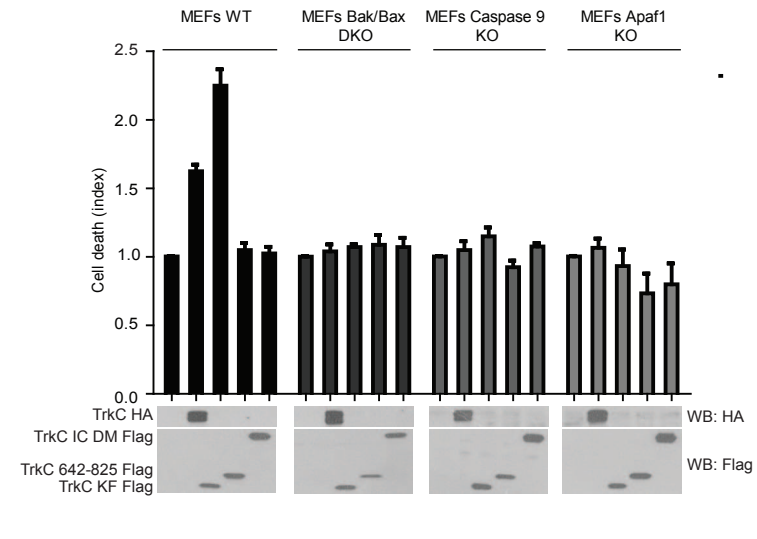
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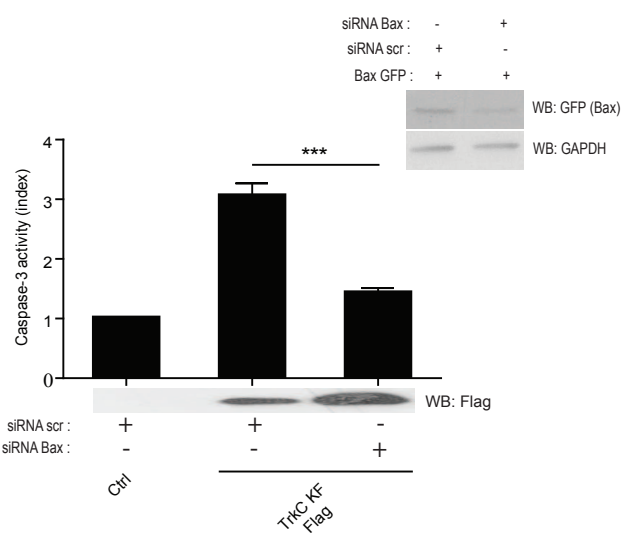
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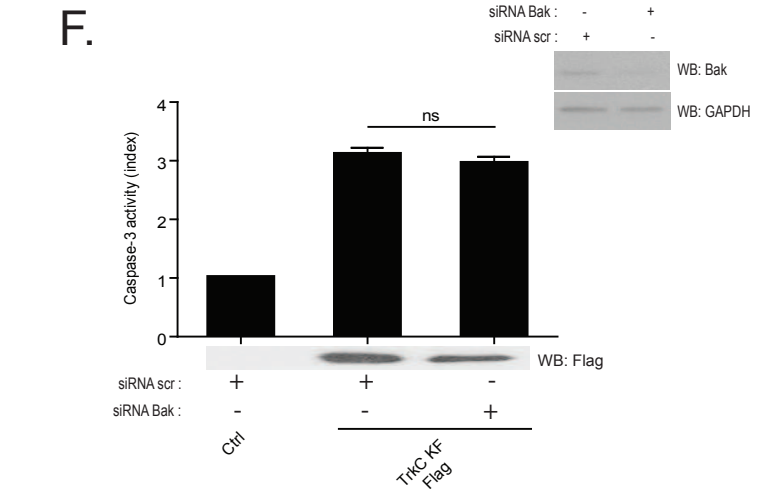
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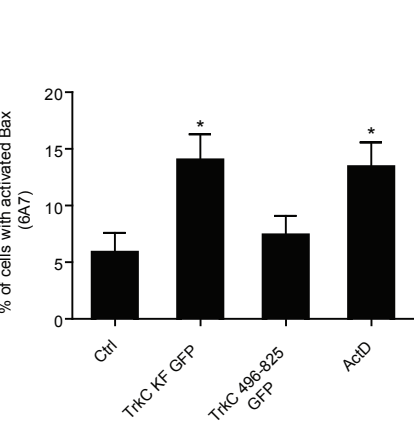


Figure 4

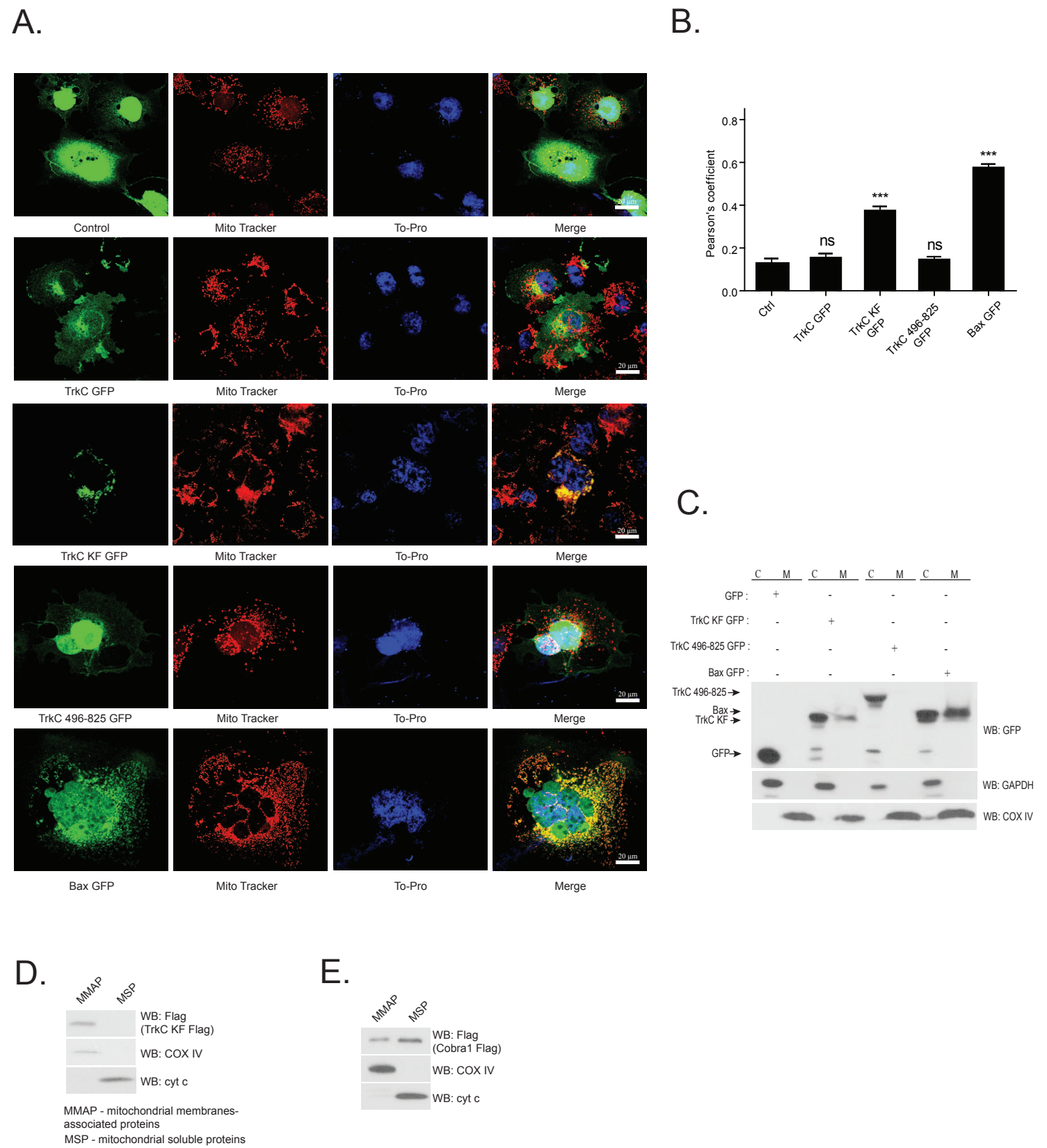


Figure 5

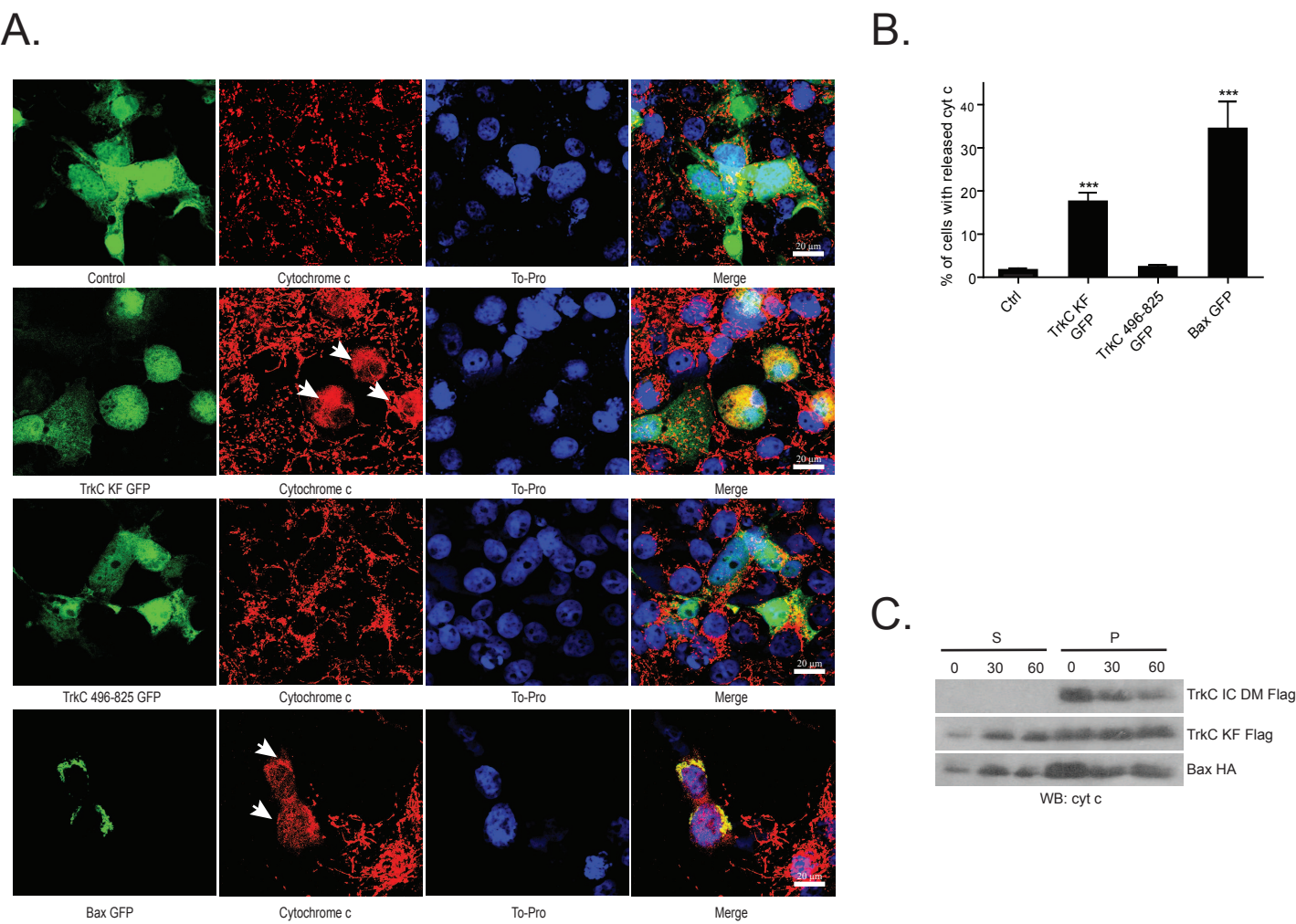
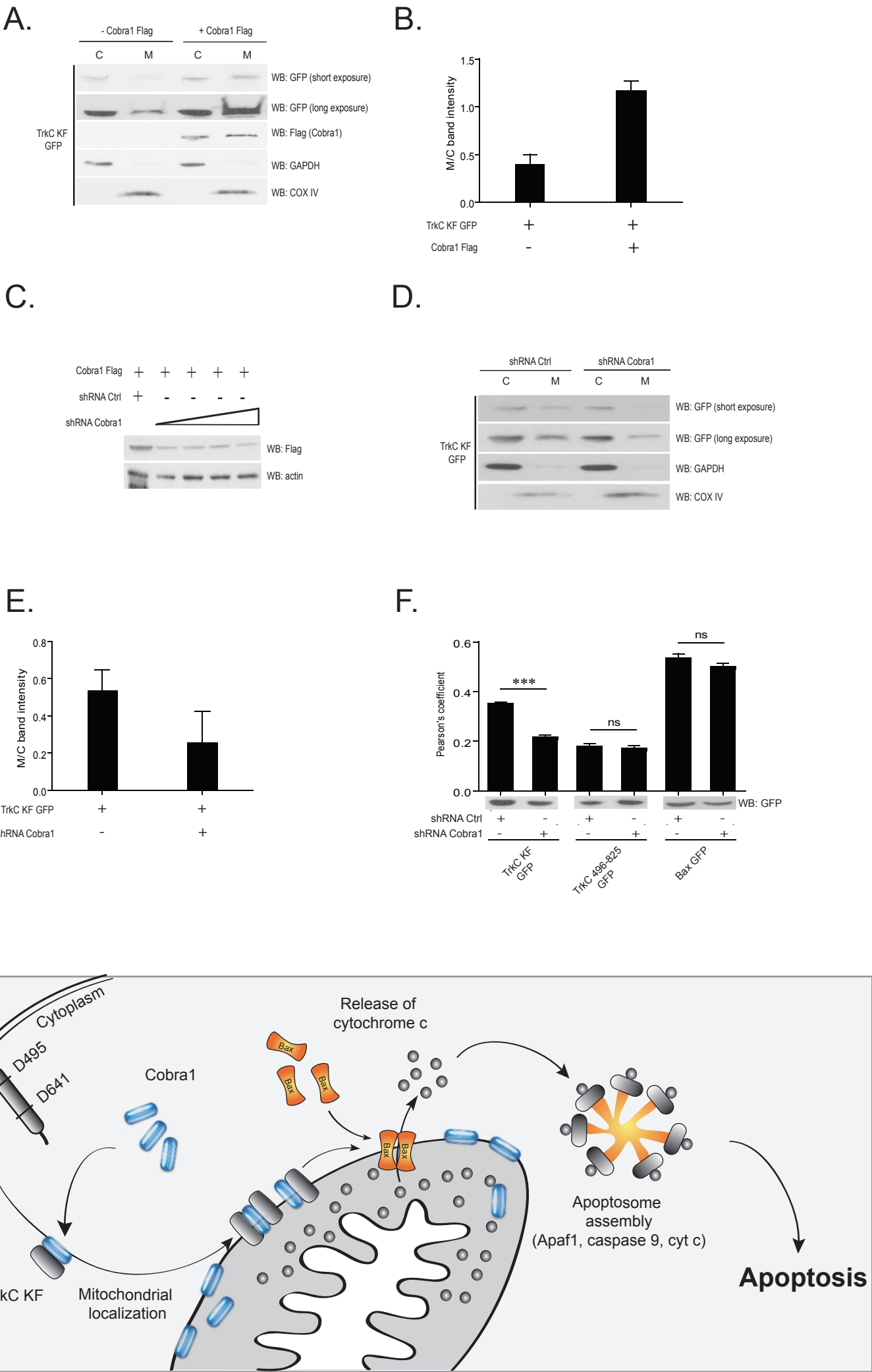
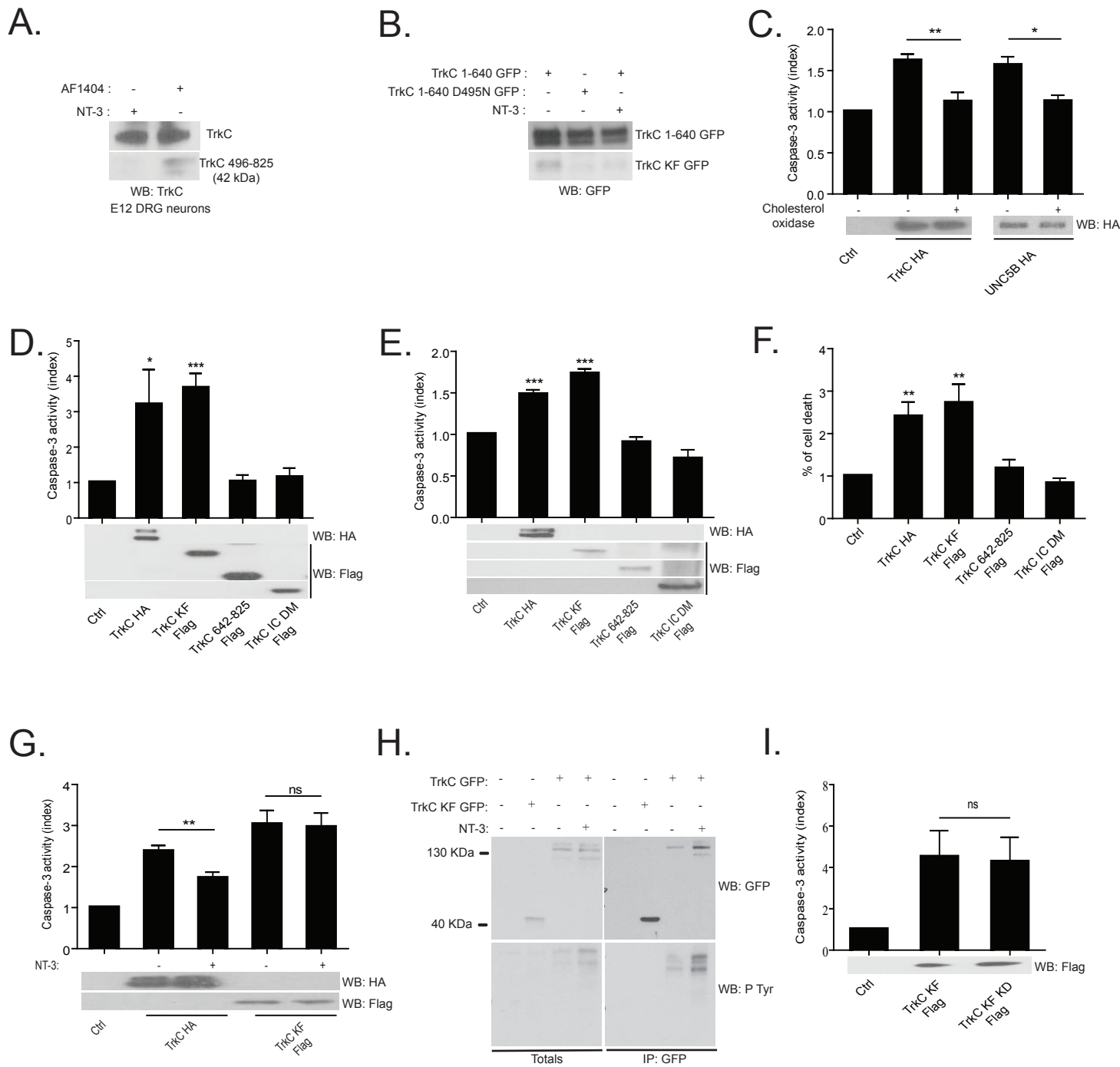


Figure 6

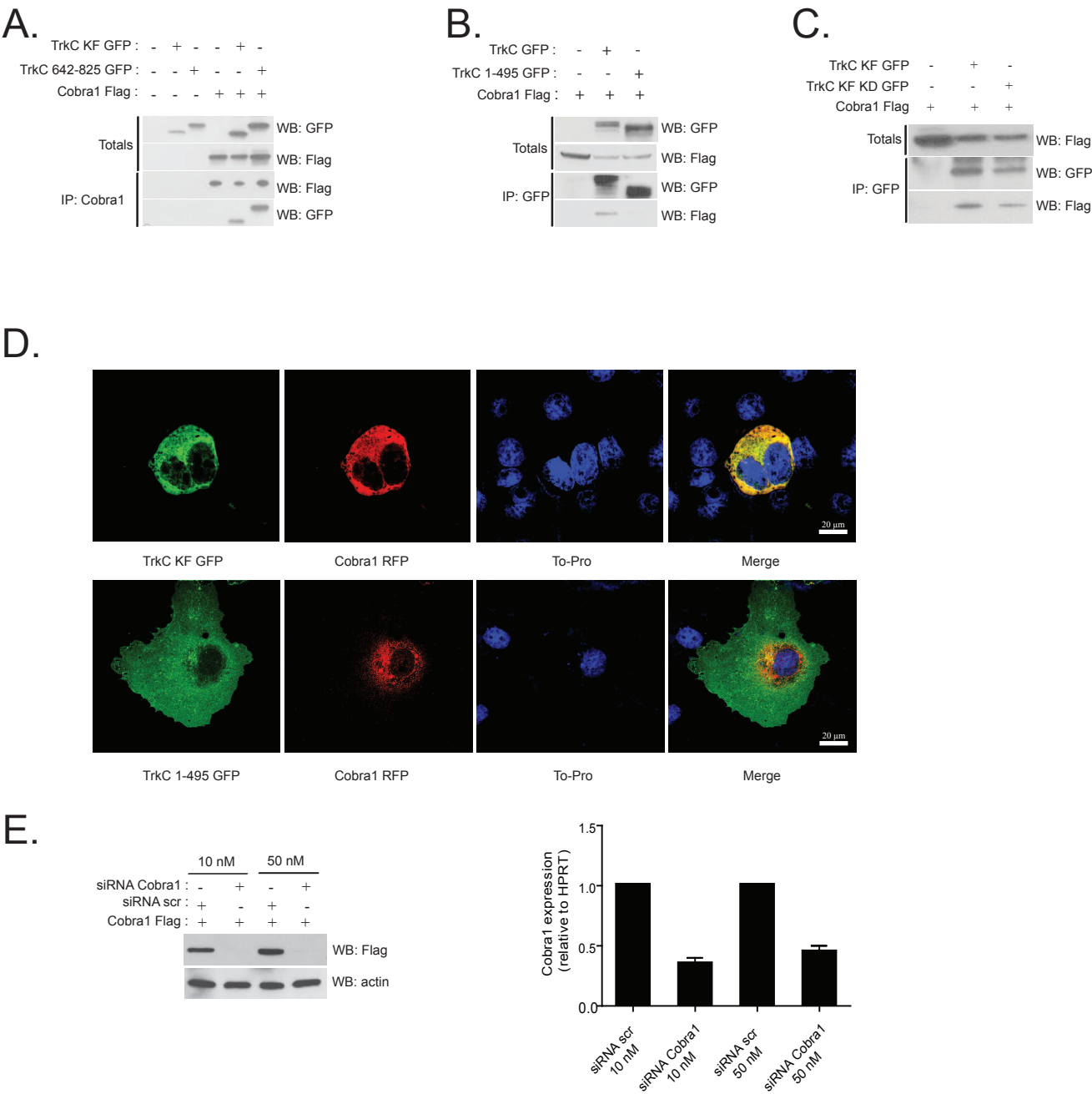


# Supplementary Figure 1



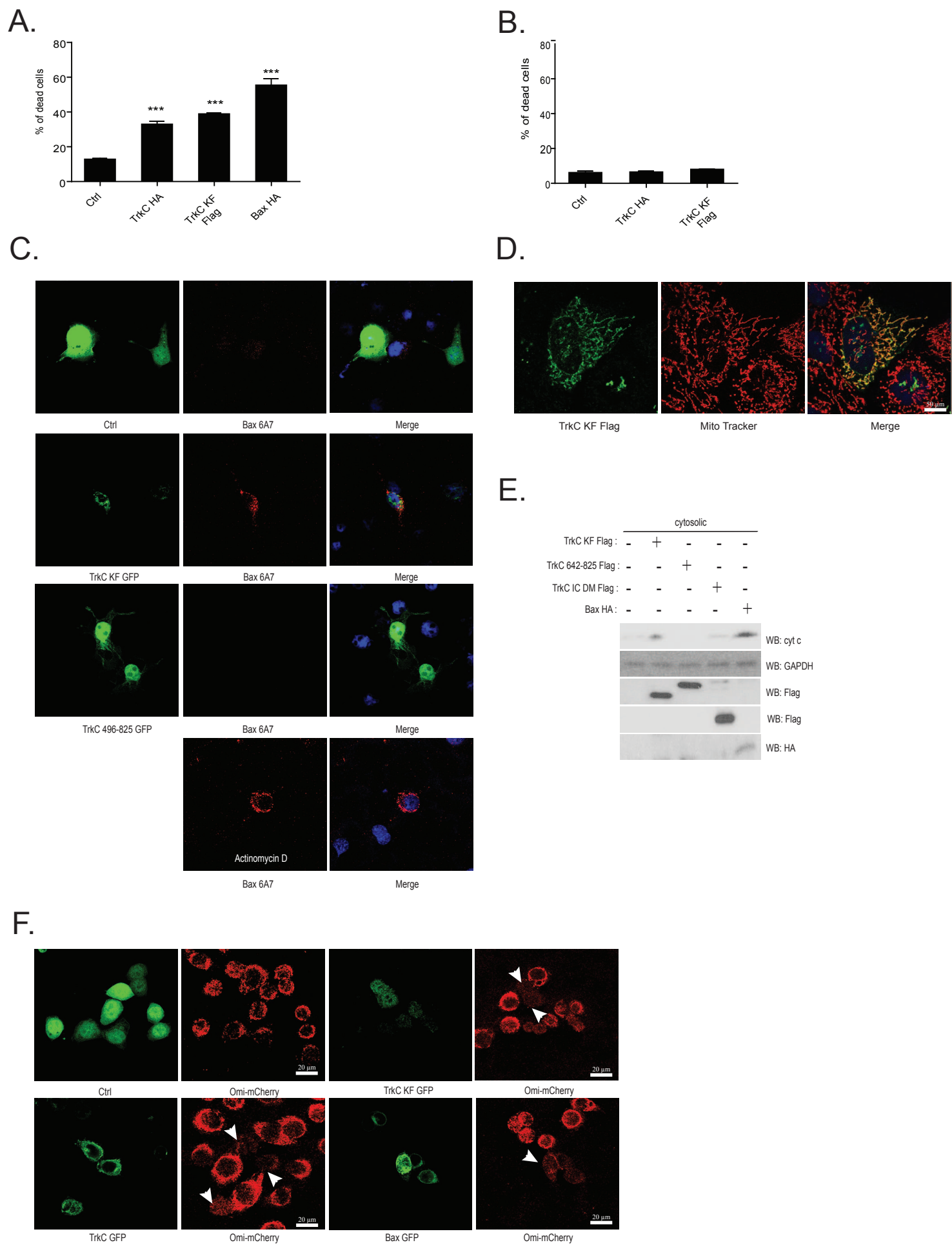


# Supplementary Figure 2





Supplementary Figure 3



## **2.2 Article 2: Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TRKC-induced apoptosis**

The analysis of a large cohort of aggressive neuroblastoma tumors revealed that almost 40% of the tumors have an increased production of NT-3. This was also characteristic for a subset of neuroblastoma cell lines, most of them derived from stage 4 tumors.

Since one of the strategies employed by tumor cells to evade TRKC-induced apoptosis might be producing in excess their own ligand, we next investigated if this was the case in neuroblastoma. Along this line, we have invalidated NT-3 in several cell lines over-expressing the ligand and noticed a re-induction of apoptosis. Similar results were obtained when using a TRKC-blocking antibody (AF1404) that prevents the NT-3/TRKC binding.

We have finally validated our hypothesis *in vivo*, in both avian and murine tumorigenesis models. First, we developed a neuroblastoma tumorigenesis model using chick embryos: shortly, the cancer cells are inoculated on the chorioallantoic membrane in the egg; here they form a primary tumor from which cell will disseminate to a secondary site *i.e.* lungs. Remarkably, in this model treatment with both siRNA for NT-3 and AF1404 resulted in tumor growth inhibition. In parallel, we xenografted NT-3 expressing cells in nude mice and treated the resulting tumors with siRNA for NT-3. We have observed in this model a reduction of tumor size when treated with the NT-3 siRNA, compared with the scrambled siRNA treatment. In this study I was doing mainly death assays on neuroblastoma cell lines and I also was involved in the murine xenograft experiments.

To conclude, we have established the proof of concept that the tumor suppressor role of TRKC is inhibited in neuroblastoma by an autocrine expression of NT-3 and we validated basic therapeutic strategies centered on the disruption of TRKC/NT-3 interaction.



# Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TrkC-induced apoptosis

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**Tropomyosin-related kinase receptor C (TrkC) is a neurotrophin receptor with tyrosine kinase activity that was expected to be oncogenic. However, it has several characteristics of a tumor suppressor: its expression in tumors has often been associated with good prognosis; and it was recently demonstrated to be a dependence receptor, transducing different positive signals in the presence of ligand but inducing apoptosis in the absence of ligand. Here we show that the 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. Functionally, both siRNA knockdown of NT-3 expression and incubation with a TrkC-specific blocking antibody triggered apoptosis in human NB cell lines. Importantly, disruption of the NT-3 autocrine loop in malignant human neuroblasts triggered in vitro NB cell death and inhibited tumor growth and metastasis in both a chick and a mouse xenograft model. Thus, we believe that our data suggest that NT-3/TrkC disruption is a putative alternative targeted therapeutic strategy for the treatment of NB.**

## Introduction

According to the classic neurotrophic theory, neuronal survival is dependent on neurotrophic factors, such as neurotrophins (1, 2). These proteins are crucial to the development of the nervous system, especially as they control the massive developmental loss of excess neurons that fail to adequately connect to their targets. However, over the years, neurotrophins — that include NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5 (2) — and their tropomyosin-related kinase receptors — TrkA, TrkB, and TrkC — have been shown to play an important role in cancer (reviewed in ref. 3). The overall view is that, as is the case with classic tyrosine kinase receptors, dysregulation of kinase activities through various mechanisms generates survival signals via the PI3K/Akt and Ras/MEK/MAPK pathways, which in turn promote tumor progression (4).

However, TrkC does not seem to fit this picture: (a) its expression is a good-prognosis factor in different types of cancer and more specifically in melanomas (5), medulloblastomas (6, 7) and neuroblastomas (NBs) (8–12); (b) its expression and activation have been shown to trigger apoptosis in medulloblastoma cells (13); and (c) as opposed to TrkA and TrkB, it was recently proposed to act as a dependence receptor (14). Such receptors are able to initiate 2 completely opposite signaling pathways, depending on ligand availability. In the presence of ligand, a positive differentiation, guidance, or survival signal is transduced. In the absence of ligand, however, they induce an active process

of apoptotic cell death. These dependence receptors also include p75<sup>NTR</sup>, deleted in colorectal cancer (DCC), UNC5H, Patched, and neogenin and the RET, EPHA4, and Alk tyrosine kinase receptors (15–20). The proapoptotic activity of dependence receptors is believed to be important for adequate neuron migration or localization during the development of the nervous system (21–23) but also for inhibiting tumor growth.

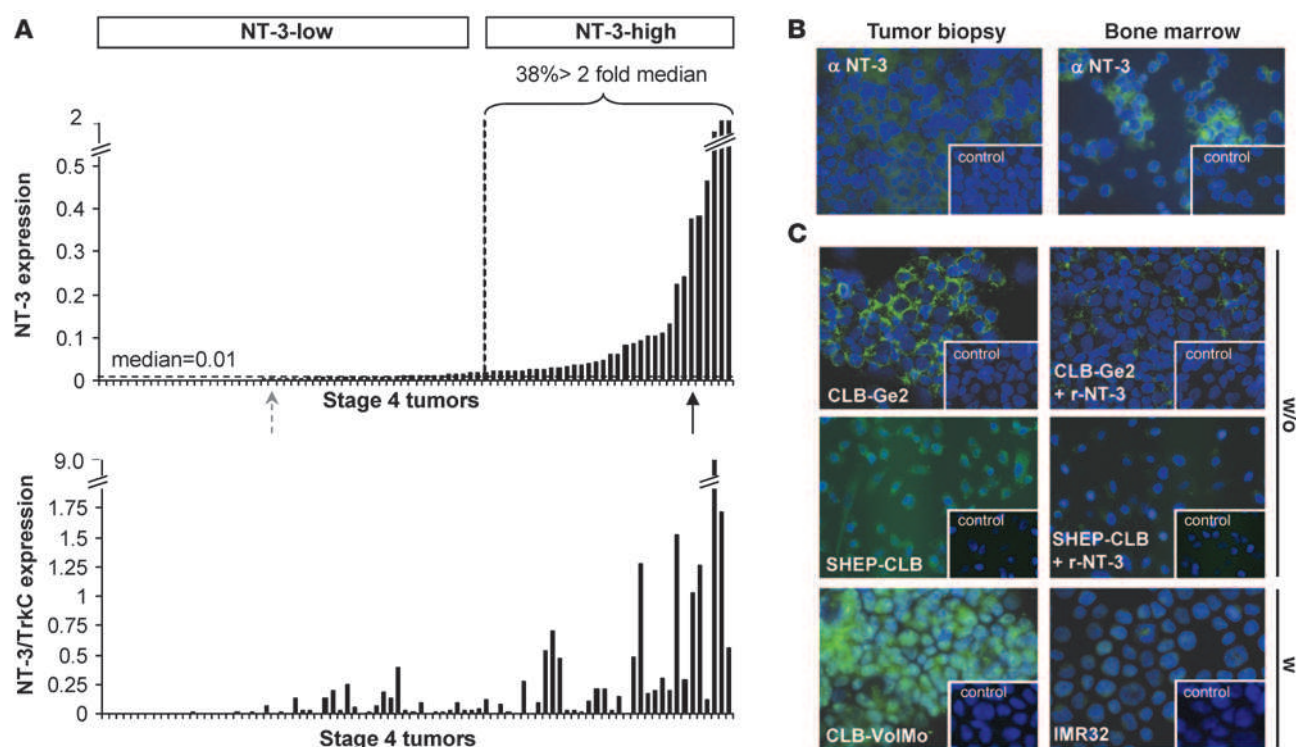
Along this latter line, this dependence on ligand presence is also thought to act as a safeguard mechanism to prevent tumor cells from developing in settings of ligand unavailability (reviewed in refs. 24, 25). This view is supported by the fact that in mouse gastrointestinal tracts, disrupting the proapoptotic signaling of a specific dependence receptor by overexpressing its ligand (netrin-1) or inactivating the receptor itself (UNC5H3) is associated with intestinal tumor progression (26, 27). Thus, a tumor losing a dependence receptor’s proapoptotic activity would gain a selective advantage for growth. This can be achieved by 3 possible mechanisms: (a) loss or decrease of expression of the dependence receptor itself as extensively described for DCC (28) and more recently for UNC5H (25, 27), (b) inactivation of the death signaling pathway, and (c) autocrine production of the ligand. The latter is of great interest in terms of potential therapy, as extracellular interference between ligand and receptor could drive a tumor cell to apoptosis.

We describe here that a large fraction of aggressive NBs that express TrkC has evolved toward a gain of NT-3 expression. NB, the most frequent solid tumor in young children, is diagnosed as a disseminated (stage 4) disease in more than 50% of cases. NB prognosis is highly variable depending on ploidy, pathology, and MYCN gene amplification (reviewed in ref. 29). Current treatments are often limited and induce deleterious side effects. Therefore, new treatments need to be developed that are more tumor specific,

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**Figure 1**

NT-3 is expressed in a large fraction of stage 4 NBs. (A) NT-3 expression and NT-3/TrkC ratio measured by Q-RT-PCR on total RNA from tumors from a total of 86 stage 4 NB patients. The percentage of tumors expressing NT-3 at more than 2 fold of the value corresponding to the median is indicated. NT-3-low, tumors with low levels of NT-3 expression; NT-3-high, tumors with high levels of NT-3 expression. (B) Representative NT-3 immunohistochemistry on a tumor biopsy and bone marrow dissociated cells from low (left panel) and high (right panel) NT-3-expressing stage 4 patients, corresponding to the dotted gray arrow and black arrow in A, respectively. (C) Representative NT-3 immunohistochemistry on CLB-Ge2, SHEP-CLB, CLB-VolMo, and IMR32 cells. CLB-Ge2 and SHEP-CLB NT-3 immunostaining, when an excess of recombinant NT-3 (r-NT-3) is added with primary antibody, is also shown. Note that the 4 top panels show immunohistochemistry performed in absence of membrane permeabilization (w/o), while the immunohistochemistry shown in 2 bottom panels was performed after cell permeabilization with Triton X-100 (w). (B and C) Insets depict control without primary antibody. Original magnification,  $\times 32$ .

and consequently, it is key to identify new targets for drug development. We show here that NT-3 expression in NB cells is a selective advantage for tumor cell survival and propose the disruption of the NT-3/TrkC interaction as a novel anticancer strategy to our knowledge in NBs with high NT-3 levels.

## Results

We focused on stage 4 NBs, with a specific interest in comparing the expression levels of NT-3 and its receptor TrkC. We first analyzed the expression of NT-3 and TrkC using quantitative RT-PCR (Q-RT-PCR) in a panel of 106 stage 4 NB tumors. A substantial fraction of stage 4 NBs displayed high NT-3 levels (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI41013DS1). Thirty-eight percent of tumors showed at least a 2-fold increase in NT-3 expression compared with the median value, and more than 20% of tumors displayed a 5-fold increase (Figure 1A). Tumors with high NT-3 level showed a high NT-3/TrkC ratio, supporting the view of a gain of NT-3 expression in tumors (Figure 1A). We then compared NT-3 levels to the prognosis within the different subcategories of stage 4 NBs – stage 4 either diagnosed before 1 year of age or later and stage 4S. No significant differences were observed, suggesting that NT-3 upregulation is a selective gain that occurs inde-

pendently of tumor aggressiveness and dissemination in a large fraction of stage 4 NBs. Similar results were obtained on stage 1, 2, or 3 NBs (Supplemental Figure 1A). Expression of NT-3 was not only detected at the mRNA level but also at the protein level by immunohistochemistry (Figure 1B).

NT-3 overexpression was seen in 38% of stage 4 NBs but also in a fraction of NB cell lines mainly derived from stage 4 NB tumor material (Table 1). Four human NB cell lines, i.e., CLB-Ge2, CLB-VolMo, SHEP-CLB, and IMR32, were studied further. All 4 cell lines express TrkC (data not shown), but CLB-Ge2, CLB-VolMo, and SHEP-CLB express high levels of NT-3, whereas NT-3 was barely detected in IMR32 cells both at the messengers level (Table 1) and at the protein level (Figure 1C). Interestingly, NT-3 immunostaining performed on CLB-Ge2 and SHEP-CLB cells, in the absence of cell permeabilization, showed a clear membranous staining. This suggests that the high NT-3 content observed in aggressive NB is associated with an autocrine expression of NT-3 in NB cells. We then started investigating the mechanisms allowing NT-3 a high level of expression in these cells. We failed to detect *Ntf3* gene amplification or enhanced transcriptional activity of the NT-3 promoter in these cells (data not shown). However, we noticed that inhibition of methyltransferases with the inhibitor 5-aza-2'-deoxycytidine in either CLB-Ge2 or IMR32 cells spe-



**Table 1**

NT-3 expression level and NT-3/TrkC ratio in cell lines derived from NBs

Cell line	NT-3 ( $10 \times 10^{-3}$ )	NT-3/TrkC
<b>Low NT-3 expression</b>		
IGRN91	0.01	0.0007
<b>IMR32</b>	<b>0.02</b>	<b>0.0000</b>
CLB-Es	0.06	0.0002
CLB-BARREC	0.09	0.0007
CLB-BouLT	0.10	0.0017
CLB-Hut	0.13	0.0002
CLB-Ber2	0.15	0.0007
CLB-Ma2	0.15	0.0008
SKNAS	0.18	0.024
CLB-Ber1	0.20	0.0003
CLB-Tra	0.27	0.0016
CLB-Bel	0.28	0.0002
CLB-Re	0.32	0.0010
SHSY 5Y	0.36	0.2887
<b>High NT-3 expression</b>		
CLB-Ma1	0.57	0.01
CLB-Bar	0.62	0.04
CLB-Pe	0.62	0.005
<b>SHEP-CLB</b>	<b>0.74</b>	<b>2.9</b>
CLB-Ga	0.75	0.33
CLB-Sed	1.02	0.006
CLB-Ge1	3.04	0.01
CLB-Ca	5.47	0.02
<b>CLB-Ge2</b>	<b>8.01</b>	<b>0.14</b>
<b>CLB-VolMo</b>	<b>9.04</b>	<b>0.02</b>

NT-3 expression and NT-3/TrkC ratio measured by Q-RT-PCR in a fraction of NB cell lines. HPRT expression was used as an internal control. Number of molecules as calculated by  $2^{\text{exp}(\Delta\text{Cp})}$ , where  $\Delta\text{Cp}$  is TrkC or NT-3 Cp minus HPRT Cp. Values obtained were divided by 1,000. Bold indicates cell lines further used in this study.

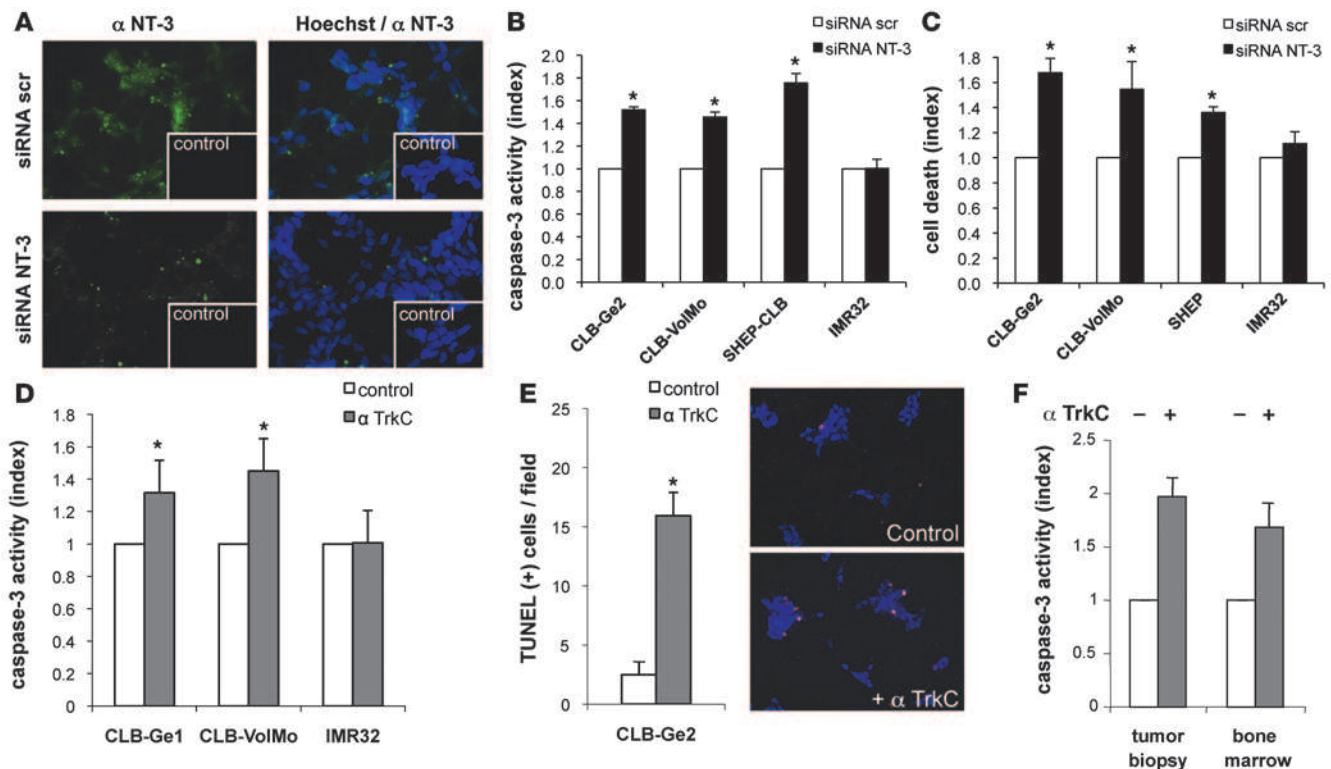
cifically restored NT-3 expression in IMR32 cells (Supplemental Figure 1B). Together with the general hypomethylation associated with cancer, this observation suggests that NT-3 high expression is associated with inhibition of NT-3 promoter methylation.

To investigate whether the NT-3 autocrine expression observed in CLB-Ge2, CLB-VolMo, and SHEP-CLB cells provides a selective advantage for tumor cell survival, as would be expected from the dependence receptor paradigm, cell death was analyzed in response to the disruption of this autocrine loop. As a first approach, NT-3 was downregulated by RNA interference. In this approach, we used either a mix of 3 siRNA sequences (siRNA NT-3) or 2 of them independently (siRNA NT-3-a and siRNA NT-3-b). Efficacy of NT-3 siRNAs tested was evaluated at the *Ntf3* mRNA level by Q-RT-PCR (Supplemental Figure 2, A and B) and at the NT-3 protein level by immunohistochemistry (Figure 2A) or by ELISA performed on the conditioning medium (Supplemental Figure 2A). While scrambled siRNA failed to affect cell survival in every cell line studied, as measured by caspase activity (Figure 2B) or ToxiLight (Figure 2C) assays, the transfection of NT-3 siRNAs was associated with CLB-Ge2, CLB-VolMo, and SHEP-CLB cell death (Figure 2, B and C, and Supplemental Figure 2C). In contrast, IMR32 cell survival was unaffected after NT-3 siRNA treatment (Figure 2, B and C, and Supplemental Figure 2C).

As a second approach, we used a blocking TrkC antibody described before (14) to prevent NT-3 from binding to endogenous TrkC. As shown in Figure 2, D and E, and Supplemental Figure 2D, the addition of anti-TrkC triggered CLB-Ge2 and CLB-VolMo apoptotic cell death, as measured by caspase-3 activity assay (Figure 2D) and TUNEL staining (Figure 2E and Supplemental Figure 2D). This effect was specific for NT-3/TrkC inhibition, since the anti-TrkC antibody had no effect on IMR32 cells. Similar CLB-Ge2 cell death induction was observed when, instead of using a blocking TrkC antibody, a recombinant ectodomain of TrkC was used to trap NT-3 (Supplemental Figure 2E).

To determine whether the NB cell death associated with inhibition of the TrkC/NT-3 interaction can be extended to fresh tumors, a surgical biopsy from a tumor and the corresponding bone marrow was semi-dissociated and further incubated with the anti-TrkC antibody. This primary tumor and the disseminated neoplasia expressed both NT-3 and TrkC (Supplemental Figure 2F), and an increased cell death, measured by caspase activation, was detected in response to the anti-TrkC antibody (Figure 2F).

There are 2 different paradigms for interpreting cell death associated with the interference of the NT-3/TrkC interaction. According to the classic neurotrophic view, the observed cell death could be a death by “default” that results from the loss of survival signals triggered by the NT-3/TrkC interaction — i.e., MAPK or PI3K pathways activated through TrkC’s kinase activity. The dependence receptor method offers a different perspective, more compatible with the fact that TrkC expression is usually a good prognosis factor. In this scenario, blocking the interaction between NT-3 and TrkC leads to unbound TrkC actively triggering apoptosis. As a first approach to discriminate between these 2 possibilities, NB cell death was induced via anti-TrkC antibody treatment, after CLB-Ge2/CLB-VolMo transfection with a dominant-negative mutant for TrkC. This dominant-negative mutant, TrkC-IC D641N, has been shown to specifically inhibit the proapoptotic signaling of unbound TrkC, without affecting its kinase-dependent signaling (14). Expression of the dominant-negative mutant fully blocks anti-TrkC-mediated CLB-Ge2 and CLB-VolMo cell death (Figure 3A and Supplemental Figure 3, A and B). To further support this observation, we assessed the extent of cell death associated with NT-3 siRNA in settings of downregulation of TrkC by siRNA. As shown in Figure 3, B and C, and Supplemental Figure 3, C–E, while downregulation of TrkC in all 3 NT-3-expressing cell lines is not associated with cell death as would be expected by the classic loss of survival signaling pathways, this downregulation fully blocks cell death induced by NT-3 siRNA. This supports further the hypothesis that NT-3 upregulation observed in CLB-Ge2, CLB-VolMo, and SHEP-CLB cells inhibits the proapoptotic signaling triggered by TrkC itself. Along this line, addition of the blocking TrkC antibody to CLB-Ge2 failed to be associated with a decrease in the classic survival pathways, as exemplified here by measurement of ERK or Akt phosphorylation (Figure 3D). Moreover, TrkC caspase cleavage was enhanced by the TrkC blocking antibody (Figure 3E). Indeed, as previously described, TrkC and dependence receptors in general are cleaved by caspase, and this cleavage is a prerequisite for their proapoptotic activity (14, 16). As shown in Figure 3E, while a basal level of TrkC cleavage was detected in control conditions, addition of the blocking antibody was associated with increased TrkC cleavage, a cleavage blocked by addition of the general and potent caspase inhibitor, Boc-Asp(Ome) fluoromethyl

**Figure 2**

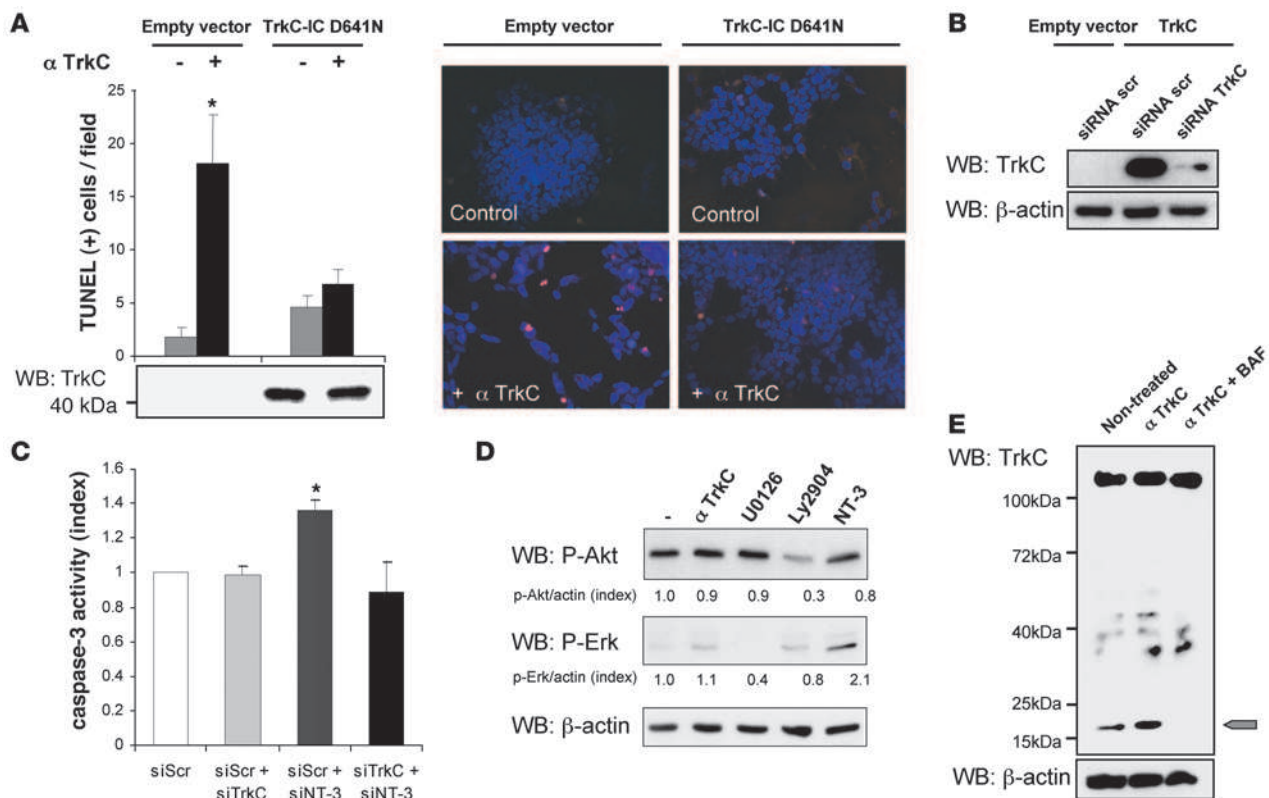
Disruption of NT-3 autocrine loop triggers NB cell death. (A) NT-3 immunostaining on the CLB-Ge2 cell line 24 hours after transfection with scrambled siRNA (siRNA scr) or with NT-3 siRNA (siRNA NT-3). Insets depict control without primary antibody. Original magnification,  $\times 32$ . (B and C) Cell death induction in CLB-Ge2, CLB-VolMo, SHEP-CLB, and IMR32 cell lines was quantified after transfection with either scrambled siRNA or a mix of 3 siRNAs targeting NT-3, using relative caspase-3 activity assay (B) or Toxilight assay (C). (D and E) Cell death induction in CLB-Ge2, CLB-VolMo, or IMR32 cell lines was quantified in cells treated with anti-TrkC blocking antibody ( $\alpha$  TrkC) or without (control) anti-TrkC antibody, using relative caspase-3 activity assay (D) or TUNEL assay (E). For the TUNEL assay, a representative labeling of TUNEL-positive cells is shown (top panel, control cells; bottom panel, cells treated with anti-TrkC blocking antibody). Original magnification,  $\times 20$ . (F) Effect of anti-TrkC blocking antibody on stage 4 NB. Tumoral cells were directly dissociated from the surgical biopsy and were plated for 24 hours in presence (+) or in absence (-) of treatment. (B–F) Data represent mean  $\pm$  SEM.  $*P < 0.05$ , 2-sided Mann-Whitney test, compared with control.

ketone (BAF). Together, these data demonstrate that NT-3 upregulation observed in NB cells inhibits the proapoptotic signaling triggered by the dependence receptor TrkC.

We next assessed whether in vivo interference with NT-3/TrkC could be used to limit/inhibit NB progression and dissemination. A chicken model was developed in which grafts of NB cells in the chorioallantoic membrane (CAM) of 10-day-old chick embryos recapitulate both tumor growth at a primary site, within the CAM, as well as tumor invasion and dissemination at a secondary site, metastasis to the lung (ref. 30 and Figure 4A). In a first approach, CLB-Ge2 or IMR32 cells were loaded in 10-day-old CAM, and embryos were treated on day 11 and day 14 with anti-TrkC or an unrelated antibody. Seventeen-day-old chicks were then analyzed for primary tumor growth and metastasis to the lung. As shown in Figure 4, B and D, treatment with the anti-TrkC antibody significantly reduced primary tumor size, specifically in CLB-Ge2-grafted CAM, while an unrelated isotopic antibody had no effect. This size reduction was associated with increased tumor cell apoptosis, as shown by an increased TUNEL staining in the tumors treated with anti-TrkC (Figure 4C). More importantly, anti-TrkC also reduced lung metastasis formation in CLB-Ge2-grafted embryos (but not in IMR32-grafted embryos), as shown in Figure 4E.

To more specifically address whether the anticancer effect of the TrkC blocking antibody was due to the reengagement of TrkC-induced apoptosis through NT-3/TrkC interaction inhibition, we first analyzed the effect of NT-3 siRNA treatment on primary tumors in the chicken model. CLB-Ge2 cells were loaded on a 10-day-old CAM. Embryos were treated, through injection in the CAM blood vessels, on day 11 and day 14 with a siRNA targeting NT-3 or scrambled siRNA. Seventeen-day-old chicks were then analyzed for primary tumor growth. As shown in Figure 4F, NT-3 siRNA treatment was associated with a significant antitumor effect. Of interest, when TrkC siRNA treatment was assessed instead of NT-3 siRNA, no effect on tumor size was observed, further suggesting that the antitumor effect seen with the blocking antibody or with NT-3 siRNA is not associated with a “classic” loss of the survival signals promoted by the tyrosine kinase activity of TrkC. On the other hand, we show that a dual treatment with NT-3 siRNA and TrkC siRNA reversed the anticancer effect observed in NT-3 siRNA-treated CAM (Figure 4F). This result thus demonstrates that NT-3 inhibition is associated with tumor growth inhibition and cell death, because it reengages TrkC-induced apoptosis.

To further provide a proof of concept that NT-3 interference may represent a putative alternative therapeutic approach, classic xeno-



**Figure 3**

NT-3/TrkC interference promotes TrkC proapoptotic activity. (A) CLB-Ge2 cells were transfected with either empty vector or with a plasmid encoding the dominant-negative TrkC-IC D641N and treated for 24 hours with or without anti-TrkC blocking antibody. Cell death was monitored by TUNEL labeling of cells plated on slides. The control panel shows TrkC-IC D641N by Western blots using anti-TrkC antibody (bottom panel). Representative images are shown. Original magnification,  $\times 20$ . (B) The efficacy of TrkC siRNA was evaluated by Western blot on nonexpressing TrkC 13.S.24 olfactory neuroblasts. Cells were transfected either with empty vector or with uncleavable TrkC D945N D641N double mutant that does not trigger apoptosis, and with scrambled siRNA or TrkC siRNA (siRNA TrkC). (C) Cell death induction in the CLB-Ge2 cell line was quantified after transfection with either scrambled siRNA (siScr), TrkC siRNA (siTrkC), NT-3 siRNA (siNT-3), or a mix of TrkC and NT-3 siRNA, using relative caspase-3 activity assay. (D) Phospho-Akt and phospho-Erk levels of CLB-Ge2 cells were monitored by Western blot after 16 hours of treatment with 2  $\mu$ g/ml anti-TrkC blocking antibody 20 nM Ly29402, 100 nM U0126, or 100 ng/ml NT-3, in absence of serum. (E) Detection of TrkC cleavage band (20 kDa, indicated by the arrow) by Western blot, using an anti-TrkC antibody on cells treated (or not) with anti-TrkC blocking antibody, with or without the general caspase inhibitor BAF. (A and C) Data represent mean  $\pm$  SEM.  $^*P < 0.05$ , 2-sided Mann-Whitney test, compared with control.

grafts of NB cells in nude mice were performed. SHEP-CLB were xenografted subcutaneously in nude mice. When the engrafted tumors reached 100 mm<sup>3</sup>, mice were treated intraperitoneally 3 times a week, either with NT-3 siRNA or with scrambled siRNA as successfully performed previously (31, 32). As shown in Figure 5, the growth of tumors engrafted in mice treated with NT-3 siRNA was strongly reduced compared with the ones engrafted in scrambled siRNA-treated mice. This tumor growth inhibition was associated with a reduction in tumor weight. Together, these data further support the antitumor effect associated with NT-3 interference in vivo.

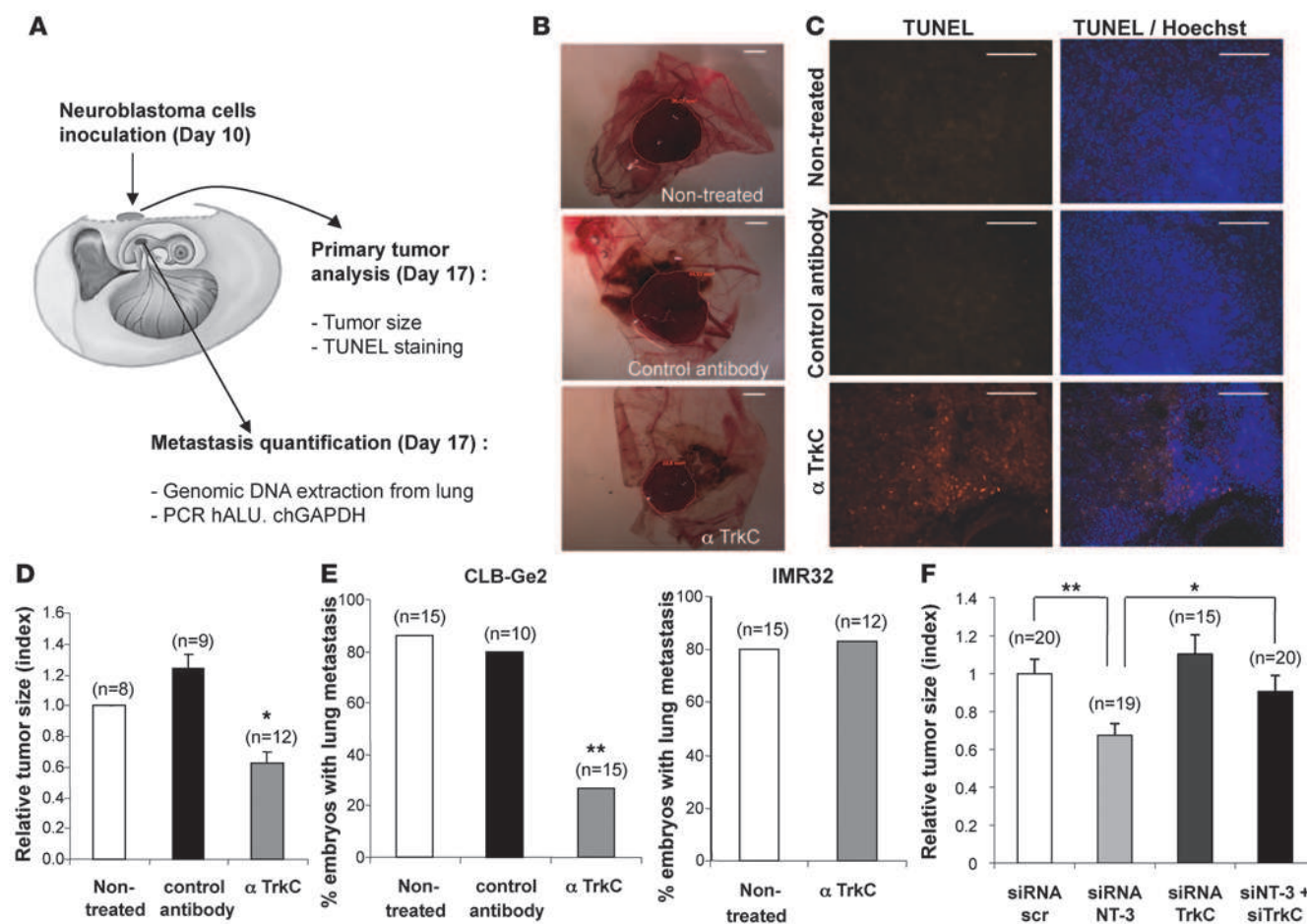
## Discussion

We show here that a fraction of NBs show an autocrine production of NT-3 associated with an increased NT-3/TrkC ratio. This elevated NT-3/TrkC ratio likely confers a selective advantage acquired by the cancer cells in settings of limited/no NT-3. Interestingly, this dependence on NT-3 presence appears specific for TrkC and does not involve other Trk receptors (i.e., TrkA or TrkB), as a dominant negative of TrkC is sufficient to turn down this dependence (Figure 3A).

Both NT-3 and TrkC have been shown to be expressed in various cancers (5, 33–47). However, most of the field has concentrated its attention on the expression of TrkC, considered as a favorable outcome factor in NB and medulloblastoma (6–8, 10–12). All these studies view NT-3 as an accessory compound that complements TrkC autoactivation and induces proliferation through TrkC kinase-dependent signaling. Along this line, NT-3 was shown to induce survival and differentiation of primary cultures derived from NB (48) and further to activate heparanase activity through TrkC and be involved in invasion during metastasis (49–51).

Here, we show that autocrine NT-3 expression is a mechanism developed by a large fraction of tumor cells to bypass TrkC-induced cell death that would occur in regions of limited NT-3 concentrations. This selective advantage is, however, only 1 of 3 possible means a tumor can use to inactivate TrkC-induced apoptosis, the 2 others being either a loss of TrkC expression or an inactivation of the downstream proapoptotic signaling. Along this later line, IMR32 cells are NT-3-negative cells and undergo apoptosis through forced expression of Bax, a classic cell death inducer.





**Figure 4**

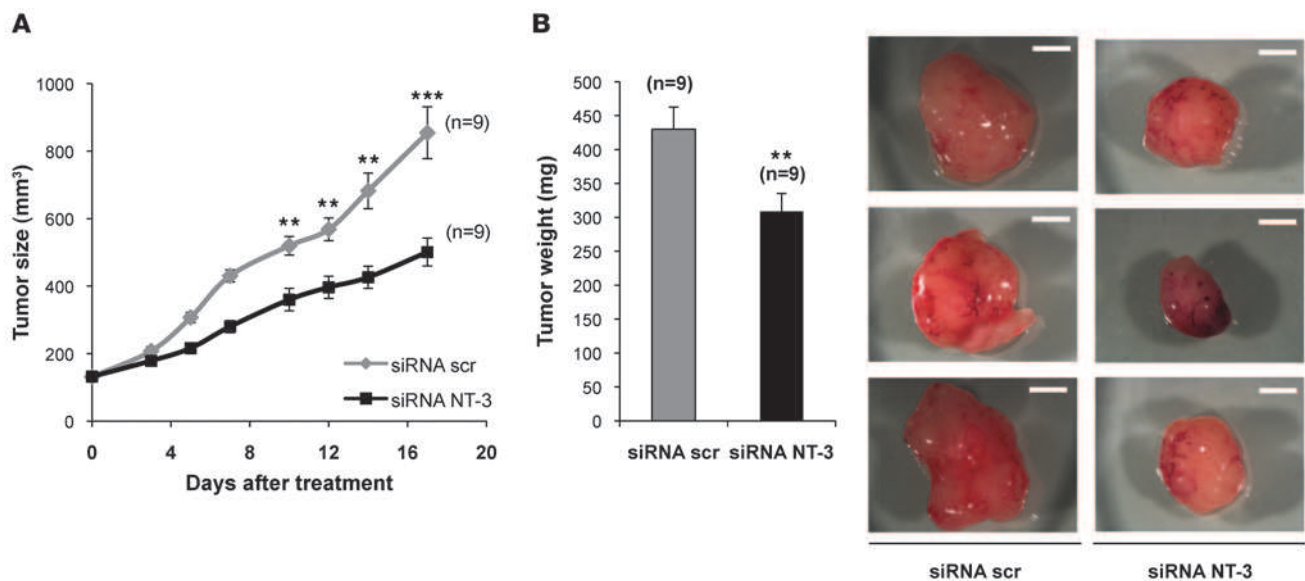
Blocking NT-3/TrkC inhibits NB growth and dissemination. (A) Schematic representation of the experimental chick model. IMR32 or CLB-Ge2 cells were grafted in CAM at day 10, and anti-TrkC blocking antibody or an isotopic antibody (control antibody) was added on day 11 and day 14. Tumors and lungs were harvested on day 17. (B–D) Effect of anti-TrkC blocking antibody on primary tumor growth and apoptosis. (B) Representative images of CLB-Ge2 primary tumors formed on nontreated CAM or CAM treated either with an isotopic antibody (control antibody) or with anti-TrkC blocking antibody. Scale bar: 2  $\mu$ m. (C) Representative images of TUNEL-positive cells in the respective primary tumors described in B. Scale bar: 100  $\mu$ m. (D) Quantitative analysis showing the primary tumor size relative to nontreated tumors. (E) Effect of anti-TrkC blocking antibody on lung metastasis. The percentage of embryos with lungs invaded by IMR32 or CLB-Ge2 cells after 2 intratumoral injections (day 11 and day 14) of either anti-TrkC blocking antibody, an isotopic antibody, or nontreated. (F) Same as for D except that NT-3, TrkC, scrambled, or NT-3 and TrkC siRNA were injected on a chorioallantoic vessel. Relative primary tumor size is presented. (D and F) Data represent mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , 2-sided Mann-Whitney test, compared with nontreated tumors. (E) \*\* $P < 0.01$ ,  $\chi^2$  test.

Nevertheless, they fail to die after TrkC overexpression (Supplemental Figure 4), thus suggesting that in IMR32 cells, the loss of TrkC proapoptotic signaling has been selected. As a consequence, NT-3 high expression constitutes a new marker for NB patients that could putatively respond to a treatment based on cell death induction via disruption of the NT-3/TrkC interaction. One may wonder, what are the mechanisms leading to NT-3 upregulation. Our preliminary results suggest that NT-3 expression in cancer cells may be regulated by epigenetic mechanisms, but further work will be needed to address this important point.

The in vitro cell death effect and the in vivo antitumor effect of NT-3 interference on NT-3-expressing tumor cells support the view that NT-3 is an exciting target for drug development. So far, the data obtained are still limited in term of the number of models tested and also on the amplitude of the anticancer effect observed (Figures 4 and 5). This, however, could be explained by the fact that

the tools used here (siRNA or polyclonal antibody) were proof-of-concept tools and definitely not drug candidate tools. Drug development has now to be explored. However, our data calls for a larger screen of cancers that could be responsive to such a therapeutic approach. Indeed, the therapeutic strategy presented here is fundamentally different from the now classic strategies for cancer therapy based on inhibitors of tyrosine kinase activity. In our view, inhibiting the kinase activity-dependent survival signals triggered by TrkC should only have poor tumor death effect, i.e., cytostatic, if the drug developed does not also target the activation of the proapoptotic activity of TrkC. We propose here that a treatment based on inhibition of the interaction between NT-3 and its dependence receptor TrkC, by blocking either NT-3 or TrkC, could potentially provide benefit, as a first line treatment or in combination with standard chemotherapy, to the large fraction of the patients suffering from aggressive NB and possibly other cancers with high NT-3 levels.





**Figure 5**

Disruption of NT-3 autocrine loop inhibits NB tumor growth. **(A)** The volume of SHEP-CLB engrafted tumors was measured during treatment with either NT-3 siRNA or scrambled siRNA. Mice were injected intraperitoneally with 3  $\mu$ g siRNA 3 times per week. Mean tumor volume is indicated. **(B)** SHEP-CLB tumors were dissected after 18 days of treatment. Final tumor weight and representative pictures are shown. Scale bar: 2 mm. Data represent mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 2-sided Mann-Whitney test, compared with scrambled siRNA-treated mice.

## Methods

**Human NB and breast tumors samples and biological annotations.** After the consent of parents, surgical human NB tumor material was immediately frozen. Materials and annotations were obtained from the Biological Resources Centers of both national referent institutions for NB treatment, i.e., Centre Léon Bérard and Institut Gustave Roussy. Approval of care and use of these samples was given by the Biological Resources Center Ethical and Steering committees of Centre Léon Bérard and Institut Gustave Roussy.

**Cell lines, transfection procedure, and reagents.** Human NB cell lines were from the tumor banks at Centre Léon Bérard and at Institut Gustave Roussy. CLB-Ge2, CLB-VolMo, SHEP-CLB, and IMR32 cell lines were cultured in RPMI 1640 Glutamax medium (Gibco) containing 10% fetal bovine serum. Cells were transfected using Lipofectamine 2000 Reagent (Invitrogen). Tumor biopsies and bone marrow cells were immediately dissociated and cultured on RPMI 1640 Glutamax medium (Gibco) containing 10% fetal bovine serum. Olfactory neuroblasts 13.S.24 were cultured and transfected as previously described (14). Anti-TrkC blocking antibody was obtained from R&D Systems (catalog AF1404). The recombinant TrkC/Fc chimera corresponding to the extracellular domain of human TrkC (Fc-TrkC-EC) was obtained from R&D Systems (catalog 373-TC). BAF caspase inhibitor (20 mM) and 5-aza-2'-deoxycytidine methylation inhibitor (5  $\mu$ M) were from Sigma-Aldrich (A3656).

**Plasmid constructs and siRNA.** The TrkC dominant-negative mutant TrkC-IC D641N and uncleavable TrkC D495N/D641N were described before (14). Scrambled siRNA (catalog sc-37007) and NT-3 siRNA (catalog sc-42125, sc-42125-A, and sc-42125-B) were obtained from Santa Cruz Biotechnology Inc. TrkC siRNA was from Sigma-Aldrich (SASI\_Hs01\_00192145 and SASI\_Hs01\_00192145\_AS).

**Cell death assays.**  $2 \times 10^5$  cells were grown in serum-poor medium and were treated (or not) with 2  $\mu$ g/ml anti-TrkC antibody (catalog AF1404, R&D Systems) and 2  $\mu$ g/ml Fc-TrkC-EC (catalog 373-TC; R&D Systems) or transfected with siRNA or TrkC constructs, using Lipofectamine 2000 (Invitrogen) for CLB-Ge2, CLB-VolMo, and SHEP-CLB cells or Lipofectamine Plus for IMR32 cells (Invitrogen). Cell death was analyzed

24 hours after treatment/transfection either by trypan blue exclusion as described previously (15) or with ToxiLight Bio Assay kit (Lonza). Apoptosis was monitored by measuring caspase-3 activity as described previously (15), using the ApoAlert CPP32 Kit from Clontech (USA). For detection of DNA fragmentation, CLB-Ge2 cells were grown in poly-L-lysine-coated slides and fixed with 4% PFA 24 hours after treatment/transfection. IMR32- and CLB-VolMo-transfected cells were cytopun before PFA fixation. TUNEL was performed with 300 U/ml TUNEL enzyme and 6  $\mu$ M biotinylated dUTP (Roche Diagnostics), as described previously (52).

**Q-RT-PCR.** To assay NT-3 and TrkC expression in NB samples, total RNA was extracted from histologically qualified tumor biopsies (>60% immature neuroblasts), using the Nucleospin RNAII Kit (Macherey-Nagel), and 200 ng were reverse transcribed using 1 U SuperScript II Reverse Transcriptase (Invitrogen), 1 U RNase inhibitor (Roche Applied Science), and 250 ng random hexamer (Roche Applied Science). Total RNA was extracted from human cell lines using the Nucleospin RNAII Kit (Macherey-Nagel), and 1  $\mu$ g was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time Q-RT-PCR was performed on a LightCycler 2.0 apparatus (Roche), using the LightCycler FastStart DNA Master SYBER Green I Kit (Roche). Q-RT-PCR was performed using the following primers: TrkC, forward 5'-AGCTCAACAGCCAGAACCTC-3' and reverse 5'-AACAGCGTTGTCACCCTCTC-3'; NT-3, forward 5'-GAAACGCGATGTAAGGAAGC-3' and reverse 5'-CCAGCCCACGAGTTTATTGT-3'. The ubiquitously expressed human *HPRT* gene, showing the least variable expression in NB, was used as an internal control, using the following primers: forward 5'-TGACACTGGCAAAACAATGCA-3' and reverse 5'-GGTCCTTTTCACAGCAAGCT-3'. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds.

**Immunohistochemistry and immunoblot.**  $8 \times 10^4$  cells were cytopun on coverslips and fixed in 4% PFA. The slides were then incubated at room temperature for 1 hour with an antibody recognizing human NT-3 (1:300;



catalog sc-547, Santa Cruz Biotechnology Inc.). After rinsing in PBS, the slides were incubated with an Alexa Fluor 488-conjugated donkey anti-rabbit antibody (Molecular Probes). Nuclei were visualized with Hoechst staining (Sigma-Aldrich).

Expression of TrkC constructs and endogenous TrkC cleavage were monitored by Western blot with anti-Trk antibody (catalog sc-11; Santa Cruz Biotechnology Inc.), and an anti  $\alpha$ -actin (13E5; Cell Signaling Technology) was used as loading control as previously described (14).

Phospho-Akt and phospho-Erk levels of CLB-Ge2 cells were measured by Western blot with anti-phospho-Akt (4058, Cell Signaling Technology) and phospho-Erk1 and Erk2 (E7028, Sigma-Aldrich) after 16 hours of culture on serum-free medium with 2  $\mu$ g/ml anti-TrkC antibody (catalog AF1404, R&D Systems), 20 nM Ly29402 (Sigma-Aldrich), 100 nM U0126 (Sigma-Aldrich), or 100 ng/ml NT-3 (Abcys).

**ELISA determination of NT-3 level.** For NT-3 determination in cell culture supernatants, 13.S.24 cells were transfected with a human NT-3 construct and scrambled siRNA or NT-3 siRNA. Supernatants were collected 24 hours later. A white 96-well plate (CORNING) was coated with a monoclonal human NT-3 antibody (MAB267, R&D systems), blocked with bovine serum albumin, and incubated with the samples, followed by detection of NT-3 by using a biotinylated anti-human NT-3 antibody (BAF267, R&D systems), a streptavidin-peroxidase polymer (S2438, Sigma-Aldrich), and a chemiluminescent substrate (Pierce ECL Western Blotting Substrate). The luminescence was read on a Tecan Infinite P500 luminometer. Human recombinant NT-3 (P450-03, Abcys) was used as standard in the ELISA.

**Chicken model for NB progression and dissemination.**  $10^7$  NB cells suspended in 40  $\mu$ l complete medium were seeded on 10-day-old (day 10) chick CAM. 2  $\mu$ g anti-TrkC antibody or an isotypic unrelated antibody (catalog sc-1290, Santa Cruz Biotechnology Inc.) were injected in the tumor on day 11 and day 14. For siRNA treatment, 3  $\mu$ g of scrambled, TrkC, or NT-3 siRNA were injected in a chorioallantoic vessel on days 11 and 14. On day 17, tumors were resected, and area was measured with AxioVision Release 4.6 software (Zeiss). To monitor apoptosis on primary tumors, they were fixed on 4% PFA, cryoprotected by overnight treatment with 30% sucrose, and embedded in Cryomount (Histolab). TUNEL staining was performed on tumor cryostat sections (Roche Diagnostics), and nuclei were stained with Hoechst. To assess metastasis, lungs were harvested from the tumor-bearing embryos and genomic DNA was extracted with the NucleoSpin Tissue Kit (Macherey Nagel). Metastasis was quantified by Q-RT-PCR detection of the human Alu sequence, using the following primers: forward 5'-ACGCCTGTAATCCCAGCACTT-3' and reverse 5'-TCGCCCAGGCTGGAGTGCA-3'. Chick GAPDH-specific primers were used as controls, using the following primers: forward 5'-GAGGAAAGGTCGCCTGGTG-GATCG-3' and reverse 5'-GGTGAGGACAAGCAGTGAGGAACG-3'. For

both couples of primers, amplification was assessed at 95°C for 2 minutes, followed by 30 cycles at 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Genomic DNA extracted from lungs of noninoculated chick embryos was used to determine the threshold.

**Xenograft of human NB cells in nude mice.** Five-week-old (20–22 g body weight) female athymic *nu/nu* mice were obtained from Charles River. Approval of care and use of mice was given by ethical committees of Centre Léon Bérard and by the French Ministry of Research. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. SHEP-CLB cells were implanted by subcutaneous injection of  $5 \times 10^6$  cells in 200  $\mu$ l PBS into the flank of the mice to make 1 tumor per mouse. When tumors reached a volume of 100 mm<sup>3</sup>, approximately 11 days after injection, 3  $\mu$ g of either scrambled or NT-3 siRNA, diluted in 300  $\mu$ l of PBS, were injected intraperitoneally 3 times per week for 18 days. Tumor sizes were measured with a caliper. The tumor volume was calculated with the following formula:  $v = 0.5 (l \times w^2)$ , where  $v$  is volume,  $l$  is length, and  $w$  is width.

**Statistics.** Data presented are representative of at least 3 independent experiments. Unless otherwise indicated, data are mean  $\pm$  SEM. Statistical significance of differences between means was assessed by a Mann-Whitney test for paired values. Categorical data was analyzed with the  $\chi^2$  test. All statistical tests were 2-sided. *P* values of less than 0.05 were considered to be statistically significant.

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### **2.3 Article 3: The dependence receptor TRKC is a putative colon cancer tumor suppressor**

In this article we analyzed the role of the dependence receptor TRKC as tumor suppressor in colon cancer. The expression of TRKC was analyzed by qRT-PCR in a panel of 45 colorectal cancer samples versus matched normal tissue, revealing a 10-fold decrease in expression in over 60% of tested tumors. This loss of expression was also found in most of the colorectal cancer cell lines analyzed.

In an effort to delineate the mechanisms responsible for TRKC down-regulation, we have analyzed by pyrosequencing the methylation status of TRKC-promoter. Interestingly, TRKC promoter shows a markedly increased methylation in colorectal tumor samples compared with normal tissue. This was also the case for the analyzed colon cancer cell lines. Of note, the treatment of cell lines with 5-aza-2'-deoxycytidine, a demethylating agent, restores the TRKC expression.

Furthermore, the re-expression of TRKC in several colorectal cell lines re-induces apoptosis and is associated with an inhibition of anchorage-dependent growth and ability to migrate. To further analyze the tumor suppressor activity of TRKC *in vivo*, HCT 116 cells forced or not to express TRKC were xenografted on the chick embryos chorioallantoic membrane. Of interest, the size of tumors derived from the TRKC-expressing cells was significantly smaller and TUNEL staining showed that these tumors have more apoptotic cells. Conversely, the presence of NT-3 fully suppresses the tumor growth inhibitory effect of TRKC, supporting the view of TRKC as a tumor suppressor in colon cancer.

In this study I performed the cell death and cell migration assays and I was also involved in setting up the tumorigenesis model in chick embryos using TRKC-expressing colon cancer cell lines.



# The dependence receptor TrkC is a putative colon cancer tumor suppressor

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**The TrkC neurotrophin receptor belongs to the functional dependence receptors family, members of which share the ability to induce apoptosis in the absence of their ligands. Such a trait has been hypothesized to confer tumor-suppressor activity. Indeed, cells that express these receptors are thought to be dependent on ligand availability for their survival, a mechanism that inhibits uncontrolled tumor cell proliferation and migration. TrkC is yet a relatively classic tyrosine kinase receptor and therefore generally rather considered as a proto-oncogene. We show here that TrkC expression is down-regulated in a large fraction of human colorectal cancers, mainly through promoter methylation. Moreover, we show that TrkC silencing by promoter methylation is a selective advantage for colorectal cell lines to limit tumor cell death. Consequently, we show that reestablished TrkC expression in colorectal cancer cell lines is associated with tumor cell death and inhibition of *in vitro* characteristics of cell transformation and *in vivo* tumor growth. Thus, TrkC appears to be a putative new colon cancer tumor suppressor.**

apoptosis | cancer | colon | dependence receptor | genetic

## Introduction

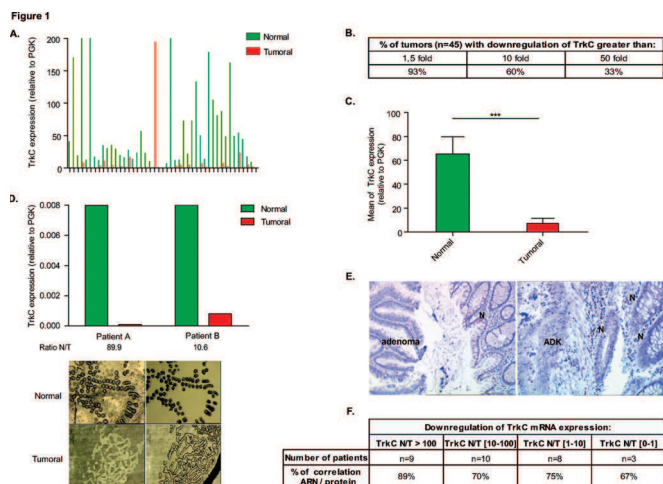
The Trk tyrosine kinase receptors and their ligands, the neurotrophins have been studied extensively for their role in nervous system development. However, TrkA was originally cloned as an oncogene from colon carcinoma tumors in which the TrkA kinase domain was fused to the tropomyosin gene in the extracellular domain (1). This discovery motivated a great number of studies which showed that neurotrophins (NGF, BDNF and NT-4/5, NT-3) and their respective Trk receptors (TrkA, TrkB and TrkC) where all involved in various malignancies (for review see(2)). The initial - and still generally accepted- view is that Trks, like other tyrosine kinase receptors are pro-oncogenic moieties and as such pan-Trk kinase inhibitors are currently being tested in clinical trials (3-5). Yet, it rapidly appeared that, at least in tumors such as neuroblastoma and medulloblastoma, TrkA, TrkB and TrkC behave differently despite their close homology. Indeed, TrkA and TrkC expression is associated with good prognosis, whereas TrkB is expressed in very aggressive tumors (for review(2)). The fact that the high expression of a tyrosine kinase receptor known to activate pro-oncogenic pathways (like the MAPK and PI3K-AKT pathways) is associated with a better outcome is counter-intuitive, and rather supports the view that TrkA and TrkC expression is a constraint for tumor progression, and that TrkA and TrkC act as tumor suppressors. Recently, two independent studies shed a new light on this paradox by demonstrating that both TrkA and TrkC, but not TrkB act as dependence receptors(6, 7).

Dependence receptors, which also include DCC (Deleted in Colorectal Carcinoma), UNC5H, Patched, Neogenin and the RET, EPHA4, IR, IGF1R and Alk tyrosine kinase receptors (8-14), share the functional property of inducing cell death when disengaged from their ligands, whereas their pro-apoptotic activity

is blocked in the presence of their ligand. These receptors thus create cellular states of dependence on their respective ligands (15, 16). The molecular mechanisms used by these unbound receptors to trigger apoptosis are in large part unknown (15, 16), but it has been suggested that this characteristic acts as a means of eliminating tumor cells that would develop in settings of ligand unavailability, such as proliferation of tumor cells in a cell environment with constant and limited ligand presence or migration of metastatic tumor cells to tissues where the ligand is absent. The pro-apoptotic activity of dependence receptors has thus been proposed to confer them a tumor suppressor activity. This was formally demonstrated for the prototype dependence receptors DCC and UNC5H3/C that bind the ligand netrin-1(16). Both DCC and UNC5C have been suggested to be colon cancer tumor suppressors because their expression is lost or massively decreased in the vast majority of colorectal cancers (17-20). Moreover inactivation of UNC5C or specific inactivation of DCC's pro-apoptotic activity in mice was shown to promote intestinal tumor progression (20, 21). Moreover, it was recently shown that missense mutations in UNC5C were associated with risk of familial colorectal cancer(22). As expected for a receptor that trigger apoptosis in settings of ligand limitation, tumor survival in settings of ligand limitation can be achieved not only by down-regulating the receptor but also by self-production of the ligand. Along this line it was shown that in several cancers such as metastatic breast cancer, lung cancer or neuroblastoma, netrin-1 is produced in an autocrine manner to block netrin-1 receptors-induced apoptosis (23-25).

Interestingly, NT-3, the ligand of TrkC was also shown to be up-regulated in neuroblastoma to block TrkC-induced apoptosis(26). Conversely, we thus investigated whether, as for netrin-1 receptors in the colon, TrkC could behave as a tumor suppressor in colorectal malignancies. We demonstrate here that expression of the *TrkC* gene is down-regulated in colorectal cancers, mainly because of tumor-associated specific promoter methylation. We also show that in colorectal cancer cell lines, re-expression of TrkC is associated with cancer cell death and consequent traits of cell transformation *in vitro*. We also show that TrkC expression is a constraint for tumor growth in an avian model. Altogether our

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**Fig. 1. TrkC expression is lost in colorectal tumors.** (A, B, C) Quantitative real-time RT-PCR was performed using total RNA extracted from normal (N) and tumoral (T) tissues with specific human TrkC primers. PGK showing the less variability in their expression between normal and colorectal tumoral tissues, as described previously (20), was used as housekeeping gene. (A) The expression levels in 45 colorectal tumors (Tumoral) and corresponding normal tissue (Normal) are given as a ratio between TrkC and PGK, the internal control. (B) Table indicating the percentage of patients showing a loss of TrkC expression in tumor compared to normal tissue. (C) Mean TrkC expression in tumoral tissues versus normal tissues is presented. \*\*\* $P < 0.001$ , 2-sided Mann-Whitney test, the two means being compared. (D) Laser capture microdissection (LCM) was performed on 8 pairs of tumor/normal tissues (2 representative pairs being presented here), and TrkC expression was determined as in (A). Top: The expression levels in 2 colorectal tumors (T) and corresponding normal tissue (N) are given as a ratio between TrkC and PGK. Bottom: typical microscopic visualization of LCM on colon section from human normal (N) or tumoral biopsies (T). Sections are shown prior to LCM (left panel), and after LCM (right panel), as the captured material is confirmed under microscopic visualization prior to processing for RNA extraction. (E) Immunostaining of TrkC protein was performed on tissue sections isolated from biopsies of 2 different patients and counterstained with Mayer's hematoxylin. N: Normal tissue, ADK: adenocarcinoma. (F) Table showing the correlation between TrkC mRNA expression and TrkC protein expression determined by immunostaining in pair normal/tumor for 30 patients in the panel of 45 patients.

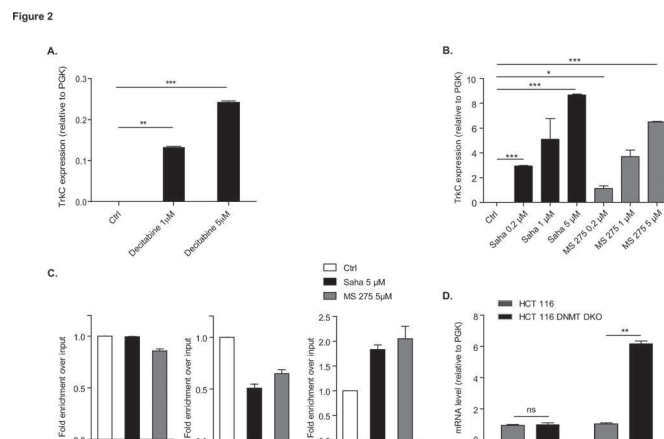
data strongly suggests that TrkC is a novel colon cancer tumor suppressor.

## Results

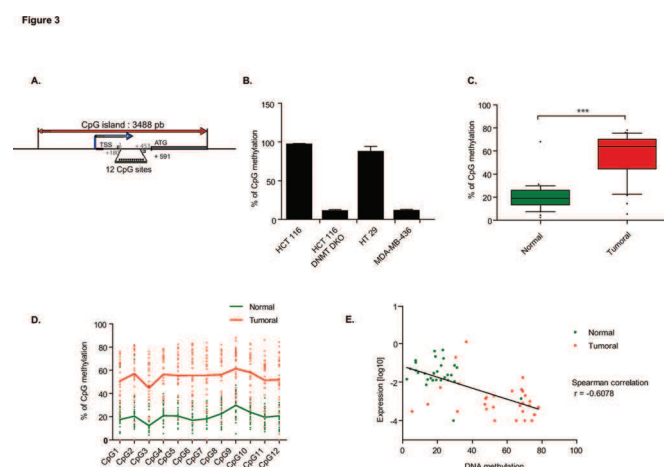
### Expression of TrkC is down-regulated in human colorectal cancers.

It had already been described that TrkC is expressed at the level of epithelial cells (27). We next assessed TrkC gene expression by quantitative real-time reverse transcriptase Q-RT-PCR in a panel of 45 colorectal cancers versus corresponding matched normal tissues. As shown in Figure 1A, while expression of TrkC was variable but relatively high in most of normal tissues, a massive decreased expression was observed in matched tumors. A 10-fold decrease of TrkC expression was observed in over 60% of the tested tumors, and a third of the tumors showed a 50-fold decrease in expression (Fig.1B). When comparing the mean TrkC expression of normal tissues versus tumors, over a 10 fold decrease was observed (Fig.1C,  $p < 0.001$ ). This strong TrkC decrease appears to be independent of the stage of the pathology, as it was similarly detected in stage I, II, III or IV colorectal cancers (Fig.S1A). Moreover, TrkC expression was reduced not only when comparing adjacent normal tissue but also normal tissues from age-matched healthy patients (Fig.S1B).

To discard a potential bias of heterogeneity in tumor/normal tissue samples, epithelial cells from tumoral/normal tissue were



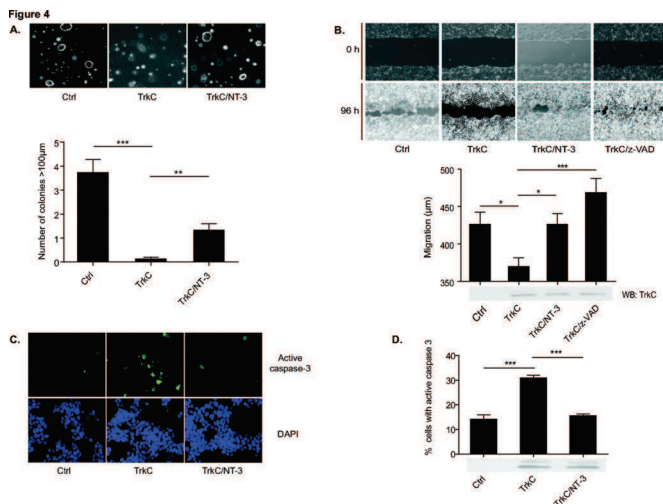
**Fig. 2. TrkC is re-expressed in HCT 116 colorectal cancer cell line following epi-drugs treatment.** (A, B) HCT 116 cells were treated for 72h with decitabine (A) or for 24h with Saha or MS 275 (B) at the indicated concentrations. TrkC expression was measured by Q-RT-PCR, using PGK as internal control. (C) Chomatin Immunoprecipitation performed on HCT 116 cells treated or not with 5 $\mu$ M Saha or 5 $\mu$ MMS275 using the following antibodies: Total H3, H3-K27Me3 (mark of transcription repression) or H4-pan-acetyl (mark of transcription activation). The experiments were done with one primers couple. Graphs show relative enrichment over Input. Enrichment for control cells is set at 1 in each individual experiment. Data are the mean  $\pm$  SEM of at least two independent experiments. (D) The level of TrkC and NT-3 mRNA was measured in wild-type (WT) and HCT 116 cells invalidated for DNMT1 and 3B (DNMT DKO) by Q-RT-PCR.



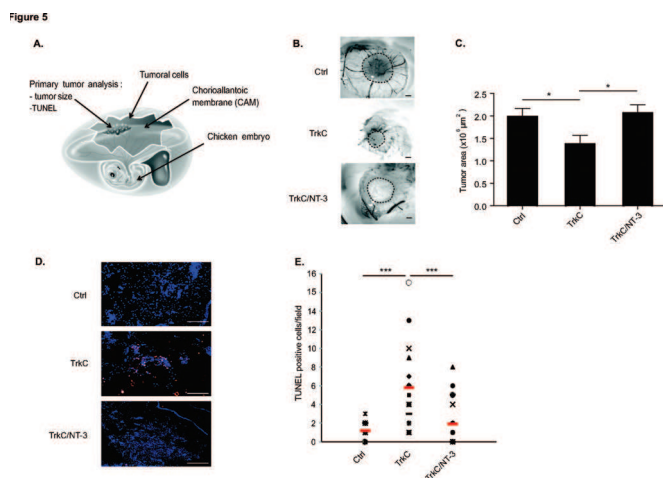
**Fig. 3. TrkC promoter is hyper-methylated in tumoral colorectal tissue.** (A) A schematic representation of TrkC promoter is shown. The main CpG island, the Transcription Start Site (TSS) and translation starting codon (ATG) are represented. The primers used for the pyrosequencing of the 12 CpG sites indicated were located in (+180;+205) and (+453;+476) (B) Analysis by pyrosequencing of the TrkC promoter methylation in TrkC negative cells (HCT 116 WT, HT29) and in TrkC positive cells (HCT 116 DNMT DKO colorectal cell lines and MDA-MB-436 breast cell line). (C) Analysis by pyrosequencing of the TrkC promoter methylation in matched normal and tumoral colorectal tissues from 30 patients in the panel of 45 patients. Statistical analysis has been performed using 2-sided Mann-Witney test. \*\*\* $P < 0.001$  (D) Pyrosequencing of the TrkC promoter methylation in matched normal and tumoral colorectal tissues from a single patient. A representative diagramme showing the methylation level on each of the 12 CpG sites analyzed is shown. (E) Inverse correlation between the decrease of TrkC mRNA level and DNA methylation of the TrkC promoter.

laser micro-dissected and assessed for TrkC expression by Q-RT-PCR. In the 8 pairs of samples assessed, an important decrease in TrkC expression was detected in tumor cells (Fig.1D, Fig.S1C).

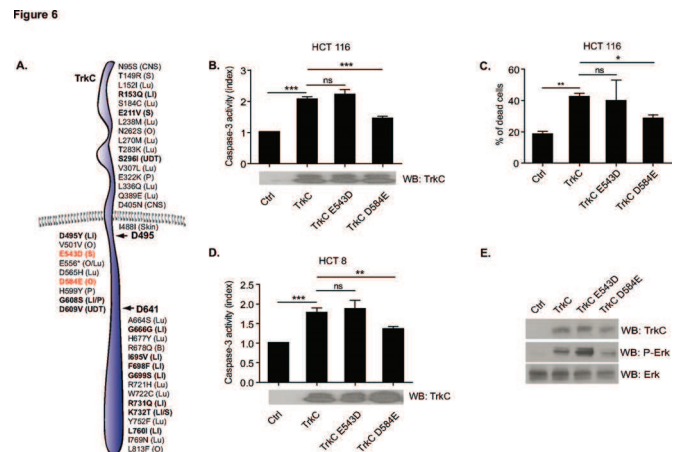




**Fig. 4. Re-expression of TrkC limits the hallmarks of colorectal cancer cells transformation via apoptosis induction.** (A) Control, TrkC or both TrkC and NT-3 overexpressing HCT 116 cells were grown in soft agar for two weeks. The number of colonies was counted in five random fields and the average number per field was calculated. Data represent mean  $\pm$  SEM.  $^{*}P < 0.05$ ,  $^{***}P < 0.001$ , 2-sided Mann-Whitney test, compared with control. Photographs of representative colonies for each condition are shown. (B) Scratch assay was performed on HCT 116 cells expressing control vector and TrkC with or without NT-3 and z-VAD-fmk addition. The scratch open area was measured at 0 hours and 96 hours and the migration distance was determined. Representative photographs are shown. (C,D) Control, TrkC or both TrkC and NT-3 overexpressing HCT 116 cells were immunolabelled for active caspase-3. Representative photographs are shown (C). The quantification and the corresponding Western-Blot controlling TrkC expression level are shown in (D).  $^{***}P < 0.001$ , 2-sided Mann-Whitney test.



**Fig. 5. TrkC expression induces tumor growth inhibition *in vivo*.** (A) Schematic representation of the experimental chick model. HCT 116 cells, transiently transfected with various plasmids, were grafted in CAM at day 10. Tumors were harvested on day 17, measured and sectioned for TUNEL staining.  $n=10$  to 15 for each condition. (B) Representative images of HCT 116 primary tumors formed. HCT 116 cells were either transfected with empty vector (Ctrl), TrkC and treated with NT-3 (10ng/ml) or not, prior to graft. Scale bar: 2  $\mu$ m. (C) Quantitative analysis showing the size of the respective primary tumors described in (B).  $^{*}P < 0.05$ , 2-sided Mann-Whitney test. (D) Representative images of TUNEL-positive cells in sections performed on the respective primary tumors described in (B). Scale bar: 100  $\mu$ m. (E) Quantification of the TUNEL-positive cells described in (D). The red bars indicate the respective mean of the various measures performed for each sample.  $^{***}P < 0.001$ , 2-sided Mann-Whitney test.



**Fig. 6. Mutations of TrkC in cancers.** (A) TrkC receptor is represented. Mutations identified in patients with various cancers are reported (<http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=NTRK3>). The caspase cleavage sites D495 and D641 are shown, they define the killer fragment of the cancer with the detected mutation is indicated. CNS: Central Nervous System; Lu: Lung; O: Ovary; P: Pancreas. Mutations detected in cancers of the gastrointestinal tract are indicated in bold (S: Stomach; LI: Large Intestine; UDT: Upper Digestive Tract). (B,C,D,E) Control, TrkC, and TrkC mutants (TrkC E543D and TrkC D584E) were over-expressed in HCT 116 and HCT 8 cells. (B,C) TrkC induces cell death in HCT 116 cells, as well as TrkC E543D but not TrkC D584E, as shown by caspase-3 activity (B) and trypan blue counting (C). (D) TrkC induces cell death in HCT 8 cells, like TrkC E543D but not TrkC D584E, as shown by caspase-3 activity. Data represent mean  $\pm$  SEM.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , 2-sided Mann-Whitney test. (E) Phospho-Erk-1/2 immunoblot is shown as an indication of Erk-1/2 activation.

The loss of TrkC in tumoral tissue observed at the RNA level was also confirmed at the protein level by TrkC immunohistochemistry on biopsy sections from 30 patients (Fig.1EF).

While TrkC level is clearly decreased in colon cancer samples, we failed to see any significant gain of NT-3 expression in these samples (Fig.S1D). This further supports that, while in neuroblastoma tumor cells appear to have selected a gain of NT-3 to survive ligand limitation(26), in colon cancer, TrkC loss is rather selected.

TrkC expression was then analyzed in a panel of colorectal cancer cell lines. As shown in Fig.S1E, most colorectal cancer cell lines screened were either negative for TrkC or showed only a modest level of expression compared to the neuroblastoma cell lines IMR32 and CLB-Ge2 in which TrkC and its ligand NT-3 were shown to be expressed (26). In this study, we used colorectal cancer HCT116 and HCT 8 cells as a model. We further confirmed that in HCT116 and HCT8 cells, TrkC was not detected by immunohistochemistry (Fig.S1F).

Taken together these data support the view that, while TrkC is expressed in the normal colon epithelium, TrkC is silenced in a large fraction of colon cancer samples and cell lines.

**TrkC expression is inhibited *via* tumor associated TrkC promoter methylation.**

In an effort to delineate the mechanisms that may explain TrkC down-regulation in colon cancer cells, we first analyzed whether loss of heterozygosity (LOH) may occur at the TrkC locus. Using a restricted panel of colorectal cancers and matched normal tissues, and a series of microsatellite markers within the *TrkC* gene, we failed to see any significant occurrence of LOH, supporting the view that TrkC down-regulation is not due to this kind of genetic event (Fig.S2AB), and suggesting an alternative mechanism for TrkC loss of expression. We then transfected a TrkC promoter-luciferase reporter construct in HCT 116 and HT29 TrkC negative cells or in MDA-MB-436 TrkC positive cells and compared luciferase expression. As shown in Fig.S2C, ex-

ogenous TrkC promoter is activated in both cell lines supporting the view that TrkC silencing is not due to indirect modulation of regulators of TrkC transcription but rather to a direct silencing of the endogenous TrkC promoter.

We thus analyzed whether TrkC expression in colorectal cancer cell lines can be restored in the presence of a DNA methylation inhibitor. As shown in Figure 2A and Figure S2D, the treatment with 5-aza-2'-deoxycytidine (Decitabine) of HCT 116 and HCT 8 cells respectively, is associated with restoration of TrkC expression. Similar results were obtained when HCT 116 or HCT 8 cells were treated with inhibitors of histone deacetylases (HDACi), such as Saha and MS275 (Fig.2B and Fig.S2E). HCT116 cells treatment with Saha or MS275 is associated with an accumulation of acetylated H4 on TrkC promoter, as revealed by Chromatin immunoprecipitation (ChIP) using an H4 pan acetylated antibody (Fig. 2C). Since, H4 acetylation is generally associated with activation of transcription(28), this observation supports the view that HDACs repress TrkC expression in HCT 116 cells. Along the same line, a decrease in the level of the repressive H3K27Me3 mark (29) was also observed at the TrkC promoter upon treatment with Saha and MS275, in accordance with the induction of its transcription (Fig.2C).

To further exclude that the data obtained above were not due to drugs toxicity, we analyzed TrkC expression in HCT 116 cells with a double knock-out for both DNMT1 and DNMT3B, two important DNA methyltransferases (30). As shown in Figure 2D, TrkC expression was increased in HCT 116 DKO DNMT1 and DNMT3b cells compared to wild-type HCT 116. Taken together, these data indicate that TrkC expression is repressed by methylation and HDAC driven deacetylation in the colorectal cancer HCT 116 cells.

We then investigated whether methylation of TrkC promoter occurs in colorectal cancer. This was assessed in a panel of 30 colorectal tumors versus adjacent normal tissues. Putative CpG islands found in the TrkC promoter (Fig.3A) were investigated for methylation by bisulfite sequencing. Following PCR amplification on the TrkC promoter (Fig.3A), pyrosequencing was used to determine the bisulfite-converted sequence of 12 CpG sites in this amplicon. The ratio of C-to-T at individual sites was determined quantitatively, based on the amount of C and T incorporation during the sequence extension (Fig.S3). In HCT 116 and HT29 colon cancer cell lines lacking TrkC, the 12 CpG sites displayed a methylation rate of 100% whereas they were not methylated in TrkC positive cells (HCT 116 DNMTs DKO and MDA-MB-436) (Fig.3B). We then analyzed TrkC promoter methylation in matched normal and tumoral colorectal tissues. The rate of methylation was remarkably increased in the tumoral samples, as compared to the normal samples (Fig.3C). We verified that the methylation was observed homogeneously on each of the 12 CpG sites analyzed (Fig.3D). We finally demonstrated a statistically significant inverse correlation between TrkC expression and the intensity of methylation (Fig.3E). Thus, TrkC expression is lost or strongly decreased in colorectal tumors through specific tumor-associated promoter methylation.

#### TrkC expression inhibits the colorectal tumor growth by triggering tumor cell apoptosis

The very frequent loss/reduction of TrkC expression in colorectal cancer suggests that the presence of TrkC is a negative constraint for colorectal tumor development. We then analyzed whether TrkC could inhibit *in vitro* the classical hallmarks of malignant transformation that are the anchorage-independent growth and the ability to migrate (31). HCT 116 cells were thus transiently transfected with a TrkC-expressing construct and allowed to grow in soft agar. Expression of TrkC receptor in HCT 116 cells was sufficient to drastically inhibit growth in soft agar (Fig.4A and Fig. S4A). Similarly, by following the closure of a scratch performed in a HCT 116 monolayer (Fig.4B), or

their migration through a transwell device (Fig.S4B), we observed that TrkC expression inhibits the migratory capacity of HCT 116 cells. This observation is somewhat surprising, as one would rather expect forced expression of a tyrosine kinase receptor to promote anchorage-independent growth and migration. Concurrently, when the same anchorage-independent growth and migration assays were performed in the presence of the TrkC ligand NT-3, the TrkC effect was antagonized (Fig.4AB and Fig.S4B). Together these data support the view that unliganded TrkC is a negative constraint for colorectal cancer cells and this observation is in agreement with the dependence receptor paradigm.

We thus assessed whether this *in vitro* tumor suppressive activity function could be related to the ability of TrkC to behave as a dependence receptor. As a first hint, we observed that upon scratch or transwell assays, the inhibitory effect of forced expression of TrkC was completely inhibited by addition of the general apoptosis inhibitor zVAD-fmk (Fig.4B and Fig.S4B). TrkC was then expressed in HCT 116 cells and cell death was determined by following caspase-3 activation either by measurement of caspase-3 substrate cleavage (Fig.S4C) or by cleaved caspase-3 immunostaining (Fig.4CD). As shown in Fig.4CD and Fig.S4C, forced TrkC expression efficiently triggers HCT 116 cells apoptosis and addition of NT-3 is sufficient to block TrkC-induced apoptosis. Similar results were obtained on HCT 8 TrkC-negative colon cancer cell lines (Fig.S4DE). Moreover, we observed that forced expression of the TrkC domain that is necessary and sufficient to induce apoptosis, the so-called TrkC Killer Fragment (TrkC KF) (6), is sufficient to inhibit anchorage-independent growth to an extent similar to that of TrkC (Fig.S4A). Together, these data support the view that TrkC is a colon cancer tumor suppressor thanks to its dependence receptor activity.

To further analyze the tumor suppressive activity of TrkC *in vivo*, HCT 116 cells forced or not to express TrkC were xenografted to the chorioallantoic membrane (CAM) of 10-day-old chick embryos (Fig.5A). The CAM of chick embryos is recognized as a convenient model to study primary tumor growth and metastasis (25, 26, 32). 17-day-old chick embryos were then analyzed for primary tumor size. As shown in Figure 5BC, the size of tumors derived from the TrkC-transfected cells was significantly smaller than tumors derived from mock-transfected cell. TUNEL staining of the tumors revealed that the inhibition of tumoral growth triggered by unliganded TrkC is probably due to its proapoptotic activity. Indeed an increased number of apoptotic cells were detected in tumors derived from TrkC expressing cells when compared to control tumors (Fig.5DE). Conversely, the presence of NT-3 fully suppresses the inhibitory activity of TrkC supporting the view that TrkC is a colon cancer tumor suppressor *in vivo* according to the dependence receptor paradigm.

#### The TrkC gene is mutated in human sporadic cancer

The view that TrkC expression down-regulation is selected in most sporadic colorectal cancers to prevent tumor cell death suggests that some sporadic cancers may rather have selected loss-of-pro-apoptotic function mutations. We therefore searched for putative *TrkC* gene mutations in the panel of tumors submitted to deep-sequencing and included in the Cosmic database (<http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&locus=1533>) or described in (33). As shown in Figure 6A, somatic missense mutations have been detected in gastrointestinal tract cancers. Of interest, several missense mutations are detected within the killer fragment of TrkC (a.a 495-641) which also encompasses part of the TrkC tyrosine kinase domain (a.a. 538-839). We thus undertook to understand whether these mutations were gain-of-function mutations constitutively inducing kinase activation as classically observed for other tyrosine kinase receptors or whether they could be, as expected according the dependence receptor paradigm, loss-of-pro-apoptotic function mutations. The different mutations shown in Fig.6A were introduced in TrkC



expressing construct and we focused on the TrkC E543D and D584E mutations that could be similarly expressed in HCT 116 cells (Fig.6B). In both HCT 116 (Fig.6BC) and HCT 8 (Fig.6D) cells, E543D mutation does not affect TrkC pro-apoptotic activity but expression of TrkC E543D mutant was shown to constitutively induce Erk-1/2 activation compared to TrkC wild-type (Fig.6E). Thus as expected for a tyrosine kinase receptor, the sporadic mutation E543D is probably a gain-of-function mutation that converts TrkC into a pro-oncogenic moiety. However when analyzing TrkC D584E, we observed that this point mutation does not affect Erk-1/2 activity (Fig.6E) but fully abrogates TrkC pro-apoptotic activity (Fig 6BCD). Together, with the fact that one of the detected mutation (TrkC E556\*) is a stop mutation deleting most of the pro-apoptotic domain of TrkC and with the fact that we failed to detect any TrkC protein production while inserting D565H and D609V mutations into TrkC, these data demonstrate that beside pro-oncogenic mutations, some mutations of TrkC turn off or decrease TrkC pro-apoptotic activity and as such behave as loss-of-function mutations inhibiting the tumor suppressive of TrkC.

## Discussion

Here we present evidence that TrkC is a colon cancer tumor suppressor, which is lost or strongly down-regulated in the vast majority of colorectal tumors. In agreement with the dependence receptor paradigm, we show here that losing TrkC and therefore surviving in settings of NT-3 limitation could be a selective advantage for a colorectal cancer cell. In the human pathology, this selective advantage appears to be achieved by promoter methylation.

According to the dependence receptor paradigm, an equivalent selective advantage to losing TrkC would be gaining autocrine expression of NT-3. However, while it was shown that in other pathologies such as neuroblastoma, NT-3 is autocrinally expressed to block TrkC-induced apoptosis(26), this does not seem to be the case in colon cancer. Why TrkC is down-expressed in some pathologies, such as colorectal cancer, while NT-3 expression is gained in others remains to be investigated. Of interest, while the detected gain of NT-3 in some tumors could be interpreted as a classic pro-oncogenic stimulation of a tyrosine kinase receptor similarly to what is seen for many other pro-oncogenic tyrosine kinase receptors (34) (35), the loss of TrkC expression in colorectal cancer definitely does not fit with the idea of TrkC being a pro-oncogenic tyrosine kinase receptor. It actually rather supports the view that TrkC is a tumor suppressor, and we present evidence both *in vitro* and *in vivo* that TrkC acts as a tumor suppressor *via* its dependence receptor function. Along the same line, expression of TrkC is a marker of favorable outcome in cancers of nervous tissue origin such as neuroblastoma (36, 37) and medulloblastoma (38, 39), in favor of the argument that in most cancers, TrkC is a stronger tumor suppressor *via* its dependence receptor than a proto-oncogene *via* its tyrosine kinase activity. This does not hold true however for the translocation resulting in the ETV6 fusion to the intracellular domain of TrkC first reported in acute myeloid leukemia with t(12;15)(p13;q25) (40) and later also observed in breast carcinoma(41), T-cell lymphoma(42) and congenital fibrosarcoma(43, 44), for which the balance between TrkC pro-apoptotic activity and pro-kinase activity is probably shifted toward the pro-kinase activity.

While the view presented here support the TrkC triggers apoptosis of cancer cell or pre-cancerous cell in setting of ligand limitation, it is interesting to look at TrkA. While TrkA has recently been shown to be a dependence receptor triggering apoptosis in the absence of ligand (7) and while preliminary data support the view that TrkA expression is also decreased in colorectal cancer, TrkA has been reported to induce apoptosis also when engaged by its ligand NGF in various cancer cell lines.

As such, NGF treatment of TrkA-expressing medulloblastoma cells induces cell death (45) while the pheochromocytoma PC12 cells equally expressing TrkA were susceptible to cell death following NGF-treatment (46). Similarly, the expression of TrkA in rat glioma cell line reduced its invasiveness in correlation with a higher rate of apoptosis (47) while TrkA-transfected U2OS osteosarcoma cells were also undergoing cell death (48). Whether TrkC is a tumor suppressor only via its dependence receptor activity or whether NT-3 contributes to this activity remains to be shown. So far we have not seen NT-3 mediated TrkC-induced apoptosis. Moreover, the absence of significant decrease of NT-3 expression in colon cancer rather supports the tumor suppressive activity of TrkC being explained by the dependence receptor paradigm.

Of interest, Bardelli *et al.* reported the spectrum of mutations within the kinase domain of tyrosine kinase proteins detected in sporadic colorectal cancer with the view that these mutations were pro-oncogenic (and gain-of-function) mutations (33). Intriguingly, TrkC was one of the most frequently mutated kinase (33). Together with the recent effort in deep-sequencing of human tumors, it appears that TrkC display a large spectrum of missense mutations. The large spectrum of mutations covering the all TrkC coding sequence (Fig.6) is a supplementary argument toward a role of TrkC as a tumor suppressor. Of interest, we now describe that, beside mutations that act a pro-oncogenic mutations (such as TrkC E543D), other mutations abrogate TrkC pro-apoptotic activity. It remains to be fully investigated if these mutations affect the generation of the pro-apoptotic TrkC killer fragment, the recruitment of pro-apoptotic protein to this killer fragment or whether it influences TrkC protein stability. Ongoing deep-sequencing will provide new mutations of TrkC in cancers and further work will demonstrate whether these mutations are oncogenic-gain-of-function mutations or, as expected according to the dependence receptor paradigm and to the data presented here, they are loss-of-pro-apoptotic-function mutations. We present data demonstrating that the TrkC dependence receptor is a colon cancer tumor suppressor and, as such, plays a similar role to DCC and UNC5H, other dependence receptors lost in colorectal cancers(20, 22, 49). Similarly, it seems that promoter methylation is a mechanism that is used by colorectal cancers to down-regulate DCC or UNC5H. This is of particular interest in the light of the currently clinically tested inhibitors of methylation or of histone deacetylases that have an anti-cancer effect (for review (50)). Whether part of this efficiency is due to reactivation of DCC, UNC5H or TrkC dependence receptor and associated apoptosis induction remains to be investigated.

## Experimental Procedures

A full description of the experimental procedures is given in Supplementary information.

### Analysis of Colorectal samples

This study is based on 90 colorectal samples from a cohort of 45 patients. To preserve anonymity, a specific ID was attributed to each patient. To assay TrkC expression in human colorectal samples, total RNA was extracted from biopsies of patients undergoing surgery for colorectal cancer. Q-RT-PCR was performed using standard procedures and primers as described in Supplementary information. Immunostaining of patient biopsies sections was performed with a anti-TrkC antibody. Sections were counterstained with a Mayer's Hematoxylin coloration.

### Cell lines and treatment

Colorectal cancer cell lines HCT 116, SW480, HT29, V9P, Caco-2, Colo320, HCT-15, SW48, SW620, SW116, SW480, ISP1, LS1034, SW837 and HCT 8 have been previously described. The HCT 116 double knock-out for DNMT1 and DNMT3b (DKO) have been previously described and were kindly provided by B. Vogelstein (30). HCT 116 cells were treated with 5-Aza-2'-deoxycytidine (Sigma-Aldrich) (1-5  $\mu$ M) 24h after plating, during 72h. HCT 116 cells were also treated with Histone DeAcetylase inhibitors (HDACi), SAHA and MS275 (Sigma-Aldrich) at a concentration range of 1-5  $\mu$ M, 24h after plating, during 24h. After treatment, cells were harvested and RNA was isolated for gene expression (Q-RT-PCR) analysis, as described above. HCT 116 transfected cells were treated with recombinant NT-3 (Abcys) at 10ng/ml, 24 h after plating.

Plasmids, TrkC site-directed mutagenesis, transfection procedure and reagents

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The full-length TrkC was already described (6). Mutations were generated on the human TrkC gene using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The plasmid constructs were transfected using JetPrime transfectant (PolyPlus) following manufacturer's instructions. The construct encoding the luciferase reporter gene under the control of TrkC promoter was a kind gift of PJ Donovan and H Fong and was described elsewhere (51).

#### Cell death assays

Apoptosis was analyzed 48 hours post transfection using caspase-3 activity assay as described before (26) while cell death index was measured using the ToxiLight assay from Lonza (LT07-117). The active caspase-3 was detected in immunostaining using an antibody specifically targeting the active form of caspase 3 (Cell Signaling, #9661).

#### Chromatin Immuno-Precipitation (ChIP) and analysis of promoter methylation

Chromatin Immuno-Precipitation experiments were performed as described in Supplementary information. The methylation status of the promoter of TrkC was examined by pyrosequencing. Bisulfite treatment was carried out as described previously (52).

#### Functional assays

Anchorage-independent growth was measured as described in Supplementary information. Transwell assays were performed as described in Supplementary information. For scratch assays, a scratch was realized with a

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pipet tip 24h later. The cell migration was monitored for the following 96h. Erk-1/2 activation was assessed as described in Supplementary information. For the chicken model for colorectal cancer progression, transfected HCT 116 cells were seeded on 10-day-old (day 10) chick chorioallantoic membrane (CAM) as described in Supplementary information.

#### Statistics

Data presented are representative of at least 3 independent experiments, data are mean  $\pm$  SEM. Statistical significance of differences between means was assessed by a Mann-Whitney test for paired values. Categorical data was analyzed with the  $\chi^2$  test. All

statistical tests were 2-sided. *P* values of less than 0.05 were considered to be statistically significant.

#### Acknowledgments:

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Classification: Biological Sciences, Medical Sciences

**The dependence receptor TrkC is a putative colon cancer tumor suppressor**

Anne-Laure Genevois et al.

**SUPPLEMENTARY INFORMATION**

- Extended Experimental Procedures
- Supplementary Figure legend and Figure 1,2,3,4.

## Experimental Procedures

### *Colorectal samples*

This study is based on 90 colorectal samples from a cohort of 45 patients. To preserve anonymity, a specific ID was attributed to each patient. For each sample, the tumoral tissue was paired with the adjacent normal tissue. The tumor stages (I-IV) were defined according to the TNM status of the tumors (American Joint Committee on Cancer (AJCC) Staging system).

### *RNA isolation & Quantitative real-time RT-PCR (Q-RT-PCR)*

To assay TrkC expression in human colorectal samples, total RNA was extracted from biopsies of patients undergoing surgery for colorectal cancer, using the Nucleospin RNAII kit (Macherey-Nagel). 500 ng of RNA were reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, France). Real-time quantitative RT-PCR (Q-RT-PCR) was performed using a LightCycler 480 (Roche Applied Science) and the FastStart TaqMan® Probe Master Mix (Roche Applied Science). Q-RT-PCR was performed using (i) probe #43 (Roche Applied Science) and the following primers: TrkC forward 5'-CAACTCTCAAACACGGAGGTC-3' and reverse 5'-CCAGCATGACATCGTACACC-3' (ii) probe #44 (Roche Applied Science) and the following primers: NT-3 forward: 5'-CCCTTGTATCTCATGGAGGATT-3' and reverse: 5'-TTTCCGCCGTGATGTTCT-3'. The ubiquitously expressed human gene PGK, showing the least variable expression between normal and colorectal tumoral tissues, was used as internal control, using probe # 42 (Roche Applied Science) and the following primers: forward 5'-

CTGTGGCTTCTGGCATACT-3' and reverse 5'-CGAGTGACAGCCTCAGCATA. For all couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 45 cycles of amplification and 30 seconds of cooling. Moreover, the cohort has been previously validated using eleven other ordinarily housekeeping genes to strengthen the results: RxR $\alpha$ , PPIA, GAPDH,  $\beta$ -actin, Phosphoglycerokinase 1,  $\beta$ 2-microglobulin, hypoxanthine ribosyltransferase, TATA-box-binding protein, porphobilinogen deaminase (PBGD), transferrin receptor, and ribosomal protein large P0 (RPLP0).

#### *Laser Capture Microdissection and Q-RT-PCR:*

Laser capture was performed under direct microscopic visualization using a Arcturus Pixcell II Laser Capture Microdissection system on 12  $\mu$ m-thick tissue frozen sections prepared from colon tumor and normal biopsies stained with cresyl violet (Ambion® LCM staining kit (Life Technologies). 3ng of RNA underwent two rounds of linear amplification using the ExpressArt® TRinucleotide mRNA amplification Nano kit, (AmpTec GmbH, Excilone). Reverse transcription of amplified RNA was performed with a iScript cDNA Synthesis Kit (Bio-Rad). cDNA synthesis was carried out from 1 $\mu$ g amplified cRNA.

Real-time Q-RT-PCR was performed on a LightCycler 2.0 apparatus (Roche), using the Light Cycler FastStart DNA Master SYBER Green I Kit (Roche). Q-RT-PCR was performed using the following primers: TrkC forward 5'-AGCTCAACAGCCAGAACCTC-3' and reverse 5'-AACAGCGTTGTCACCCTCTC-3'. The ubiquitously expressed human PGK gene, showing the least variable expression in colon, was used as an internal control, using the following primers: forward 5'-CTGTGGCTTCTGGCATACT -3' and reverse 5'- CTTGCTGCTTTCAGGACCC - 3'. For all 2 couples of primers, polymerase



was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds.

#### *LOH analysis*

The locus of TrkC has been studied with Repeat Masker. Single repeat microsatellites were used as markers to compare normal and tumor tissues using fluorescently labeled primers amplifying the DNAs from normal and tumoral tissue of 30 patients. All primers and PCR conditions are available upon request.

#### *Cell lines and treatment*

Colorectal cancer cell lines HCT 116, SW480, HT29, V9P, Caco-2, Colo320, HCT-15, SW48, SW620, SW116, SW480, ISP1, LS1034, SW837 and HCT 8 have been previously described. The HCT 116 double knock-out for DNMT1 and DNMT3b (DKO) have been previously described and were kindly provided by B. Vogelstein (1). HCT 116 Wild-Type, HCT 116 DKO colorectal cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Life Technologies), supplemented with 10% fetal calf serum (Lonza). HCT 8 colorectal cell line was grown in RPMI (Life Technologies), supplemented with 10% fetal horse serum. HCT 116 cells were treated with 5-Aza-2'-deoxycytidine (Sigma-Aldrich) (1-5  $\mu$ M) 24h after plating, during 72h. HCT 116 cells were also treated with Histone DeAcetylase inhibitors (HDACi), SAHA and MS275 (Sigma-Aldrich) at a concentration range of 1-5  $\mu$ M, 24h after plating, during 24h. After treatment, cells were harvested and RNA was isolated for gene expression (Q-RT-PCR) analysis, as described above. HCT 116 transfected cells were treated with recombinant NT-3 (Abcys) at 10ng/ml, 24 h after plating.

### *Plasmids, transfection procedure and reagents*

The full-length TrkC was already described (2). The plasmid constructs were transfected using JetPrime transfectant (PolyPlus) following manufacturer's instructions. The construct encoding the luciferase reporter gene under the control of TrkC promoter was a kind gift of Pr PJ Donovan and Dr H Fong and was described elsewhere (3).

### *TrkC site-directed mutagenesis*

Mutations were generated on the human TrkC gene using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The mutation E543D was generated with the following primers: forward 5'-GCTGAAGCGAGACCTGGGTGAGGGAG-3' and reverse 5'-CTCCCTCACCCAGGTCTCGCTTCAGC-3'. The mutation D584E was generated with the following primers: forward 5'-GCCCCGGAAGGAATTCCAGAGGGAGG-3' and reverse 5'-CCTCCCTCTGGAATTCCTTCCGGGC-3'.

### *Cell death assays*

10<sup>5</sup> cells were transfected with TrkC constructs using JetPrime (PolyPlus), and grown in serum-poor medium. Cell death was analyzed 48 hours post transfection: Caspase-3 activity was measured as described before (4) using the Ac-DEVD-AFC substrate assay (Biovision, K105-400) while total cell death index was measured using the ToxiLight assay from Lonza (LT07-117). The active caspase-3 was detected in immunostaining using an antibody specifically targeting the active form of caspase 3 and not the zymogen (Cell Signaling, #9661).

### *Protein detection*

HCT 116 and HCT 116 DKO were lysed in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA and 0.1% NP-40 in the presence of proteases inhibitors. For western blotting we used anti-panTrk (Santa Cruz, sc-139), anti-actin (Chemicon, MAB1501R).

Immunostaining of patient biopsies sections or cell lines was performed with an anti-TrkC antibody (Santa Cruz, sc-117) after citrate unmasking (pH 7.3, 98°C for 35min) and revealed with Novolink kit (Leica). Sections of patient biopsies were counterstained with a Mayer's Hematoxylin coloration.

### *DNA isolation*

DNA was isolated from 60 samples of normal and tumoral colorectal tissue from patient biopsies. All the samples were cryogrounded in liquid nitrogen and incubated at 55°C in DNA extraction buffer containing 0.1 µg/µL of proteinase K for 3 hours. After complete digestion, proteinase K was inactivated and DNA precipitated with NaCl, washed with ethanol and re-suspended in water.

### *Bisulfite conversion and analysis of TrkC promoter methylation by pyrosequencing*

The methylation status of the promoter of TrkC was examined by pyrosequencing, a highly reliable and quantitative method for the analysis of DNA methylation at multiple CpG sites with built-in internal controls for completeness of bisulfite treatment. Bisulfite treatment was carried out as described previously (5). Briefly, genomic DNA (0.5-1 µg), from associated normal and tumoral colorectal cancer samples, was treated with EZ DNA methylation-Gold kit (Zymo Research), according to the manufactured protocol.



The modified DNA (20-25 ng/μL) was stored at -20°C until use. A set of primers was designed on an in silico modified DNA sequence, to amplified a GC rich region of the TrkC promoter. DNA amplification was carried out on bisulfite-treated DNA using specific primers and PCR conditions: TrkC Forward 5'-GATTTGGTGATTTTAGTATTATTTT-3' and Reverse 5'-AAAAAAAACCTCTACCTTTAAAAC -3'. Modified DNA was amplified in a total volume of 50 μL. 10 μL of PCR was analyzed on agarose gel, and the remaining 40 μL was used in pyrosequencing assay using sequencing primers: 5'-GTTTTTAGAGTTTT-3' and 5'-TACAAAATCCTTCAA-3'. Pyrosequencing reactions were set up using PyroGold Reagent kit (Biotage, Sweden) according to the manufacturer's instructions. The methylation levels at the target CpGs were evaluated by converting the resulting programs to numerical values for peak heights and expressed either as the percentage of methylation of individual CpG sites or as the mean of all CpGs analyzed.

#### *Chromatin Immuno-Precipitation (ChIP)*

Formaldehyde was added to control or treated HCT 116 cells to a final concentration of 1%, and the cells were incubated at 37°C for 10 min. The medium was removed, and the cells were washed in 1 ml of ice-cold PBS containing protease inhibitors (Complete, Roche). Cells were pelleted, resuspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl, pH 8.1), and incubated on ice for 10 min. Lysates were sonicated with 30-sec cycles using a bioruptor. Debris were removed from samples by centrifugation for 10 min at 15,000 g at 4°C. An aliquot of the chromatin preparation (30μl) was set aside and designated as the Input Fraction. Supernatants were diluted

10-fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1, 150 mM NaCl), precleared with protein A Sepharose beads (sigma - previously blocked with salmon sperm DNA and BSA) for at least 2 hours at 4°C. Beads were pelleted by centrifugation, and supernatants incubated overnight at 4°C with 5 µg of total H3 (Abcam, ab1791), antiacetylated histone H4 (Millipore, 06-598) or anti-H3K27Me3 (Millipore 07-449) antibodies. Protein A Sepharose (40 µl) was added for 2 h at 4°C. Protein A complexes were centrifuged, washed 3 times with wash buffer (0.1% SDS, 2mM EDTA pH 8, 150mM NaCl, 20mM Tris-HCl pH8.1, 1% Triton X-100) and once with final wash buffer (0.1% SDS, 2mM EDTA pH 8, 500mM NaCl, 20mM Tris-HCl pH8.1, 1% Triton X-100). Immune complexes were eluted with 450 µl of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). Samples were treated with RNase A and proteinase K overnight at 65°C and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. DNA concentration was measured and adjusted using a nanodrop. TrkC promoter specific primers (forward: 5' GTCTTCACACTCTGTCCCCG 3' and reverse: 5' ATTTTACCCCCGTCGCCAT 3') were used to carry out Q-PCR on input and ChIP samples using Sybr green (Roche).

#### *Soft Agar Assay*

For bottom agar, 2 ml of 1.8% agarose were diluted with 2 ml of 2x DMEM (2x DMEM, 20% foetal bovine serum, 0.2% geneticin, 500 mg/ml fungizone). A 4-ml volume of bottom agar was plated in a 60-mm tissue culture dish and allowed to harden. Cells were trypsinized and resuspended at 10<sup>5</sup> cells/ml in 2X DMEM medium. The top agar cell suspensions were composed of 1 ml of cell suspension in 2X DMEM and 1 ml of

0.9% agarose, and were overlaid on dishes containing bottom agar. The final plating concentration was  $10^5$  cells per dish. 14 days later, clones were observed and counted.

#### *Migration assay (Scratch assay)*

Six well plates were coated with 50  $\mu\text{g/ml}$  poly-L-lysine by incubating the dishes overnight at 4 °C or for 2 h at 37 °C without rotation or shaking. The unbound extracellular matrix (ECM) substrate was removed and the coated dishes blocked with 3 ml of 2 mg/ml bovine serum albumin for 1 h at 37 °C. Then, the dishes were washed once with PBS and refilled with 3–5 ml of media before plating the cells.  $2 \cdot 10^6$  HCT 116 cells per well were plated and transfected with the plasmid constructs. A scratch was realized with a pipet tip 24h later. The cell migration was monitored for the following 96h.

#### *Migration assay (Transwell assay)*

Transwell migration assays were performed using 24-well transwell Boyden chambers (8  $\mu\text{m}$  PET membrane, Falcon). HCT 116 cells ( $5 \cdot 10^5$ ) were added to the top well resuspended in 500 $\mu\text{L}$  serum-free medium, and the lower well was filled with 800 $\mu\text{L}$  medium containing 20% FBS. Where applicable, NT-3 or Z-VAD was added to the top well, together with the cells. After 48-h incubation, cells remaining on the upper side of the filter were removed with cotton swabs, and the cells that had migrated to the bottom surface of the filter were counted in 10 different fields per condition. The experiment was repeated three times and one representative experiment is shown.

#### *Chicken model for colorectal cancer progression*

HCT 116 cells were transfected with plasmids 48h after plating, treated or not with NT-3 (10ng/ml) and harvested 24h post-transfection.  $5 \cdot 10^6$  HCT 116 transfected cells were suspended in 25 $\mu$ L complete medium and 25  $\mu$ L of matrigel with or without NT-3 (10ng/ml). These cells were seeded on 10-day-old (day 10) chick chorioallantoic membrane (CAM) previously locally injured with a cotton swab. On day 17, tumors were resected and the area was measured with AxioVision Release 4.6 software (Carl Zeiss, Inc).

#### *TUNEL labeling of tumor sections*

In order to monitor apoptosis on primary tumors, they were fixed on 4% PFA, cryoprotected by overnight treatment with 30% sucrose, and embedded in Cryomount (Histolab). TUNEL staining was performed on tumor cryostat sections (Roche Applied Science Diagnostics), and nuclei were stained with Hoechst (Sigma-Aldrich).

#### *MAPK pathway activation*

Cells transfected with the different TrkC mutants were lysed on ice in the following buffer (50mM Tris pH7.5, 1mM EDTA, 1mM EGTA, 0.5mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% Betamercaptoethanol, 1%Triton (x100), 50mM Sodium Fluoride, 5 mM Sodium Pyrophosphate, 10mM Betaglycerophosphate, 0,1mM PMSF). Proteins were then analysed by Western Blot using an anti-Erk antibody (Cell Signaling), an anti-phospho Erk antibody (Cell Signaling).

#### *Statistics*

Data presented are representative of at least 3 independent experiments, data are mean  $\pm$  SEM. Statistical significance of differences between means was assessed by a Mann-Whitney test for paired values. Categorical data was analyzed with the  $\chi^2$  test. All statistical tests were 2-sided. *P* values of less than 0.05 were considered to be statistically significant.

## Supplementary References

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2. Tauszig-Delamasure S, *et al.* (2007) The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. *Proc Natl Acad Sci U S A* 104(33):13361-13366.
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4. Bouzas-Rodriguez J, *et al.* (2010) Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TrkC-induced apoptosis. *J Clin Invest* 120(3):850-858.
5. Tost J, Dunker J, & Gut IG (2003) Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *Biotechniques* 35(1):152-156.

## Supplementary Figure Legends

### Supplementary Figure 1. TrkC expression is lost in colorectal tumors.

**(A,B,C)** Quantitative real-time RT-PCR was performed using total RNA extracted from normal (N) and tumoral (T) tissues with specific human TrkC and NT-3 primers. PGK was used as housekeeping gene. **(A)** The ratio of TrkC expression in normal tissue on tumoral tissue ( $\log T/N$ ) is presented for each patient, the patients being ordered by different stages (I to IV) defined by the TNM status of the tumors and by decreased loss of TrkC in tumors. **(B)** Comparison between TrkC expression in normal (green bar) and tumoral tissue (red bar) from patients and TrkC expression in age-matched tissue from healthy colon (white bar). The range of age of each group of patients is indicated in abscissa. **(C)** The level of TrkC down regulation observed after laser microdissection of tumor samples versus normal samples is indicated. **(D)** The expression levels of NT-3 ( $\times 10^{-3}$  mol) in 45 colorectal tumors (Tumoral) and corresponding normal tissues (Normal) are given as a ratio between NT-3 and PGK, the internal control. **(E)** TrkC expression ( $\times 10^{-3}$  mol) was measured in several colorectal, breast and neuroblastoma cancer cell lines, by Q-RT-PCR, using PGK as internal control. Number of molecules was calculated by  $2^{\exp(\Delta C_p)}$ , where  $\Delta C_p$  is TrkC minus PGK  $C_p$ . **(F)** Immunostaining of TrkC protein was performed on IMR32, HCT 116 and HCT 8 cells using a diaminobenzidine/peroxydase revelation.

### Supplementary Figure 2. Absence of LOH at the *TrkC* locus and TrkC re-expression in cancer cell lines.

**(A,B)** LOH analyses were performed in a panel of 30 pairs tumors and matched normal



tissues as described in the methods section. A representative pictogram of microstallite sequence amplification is shown **(B)**. **(C)** A construct encoding the luciferase reporter gene fused to TrkC promoter was transfected into HCT 116 and HT29 colon cancer cell lines and as a control into breast cancer cells, MDA-MB-436. The corresponding empty vector pGluc3 was used as control. Luciferase activity was measured in the various cell lines. **(D,E)** HCT 8 cells were treated for 72h with decitabine **(D)** or for 24h with Saha or MS 275 **(E)** at the indicated concentrations. TrkC expression was measured by Q-RT-PCR, using PGK as internal control. Data represent mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 2-sided Mann-Whitney test, compared with control.

#### **Supplementary Figure 3. Methylation of *TrkC* promoter.**

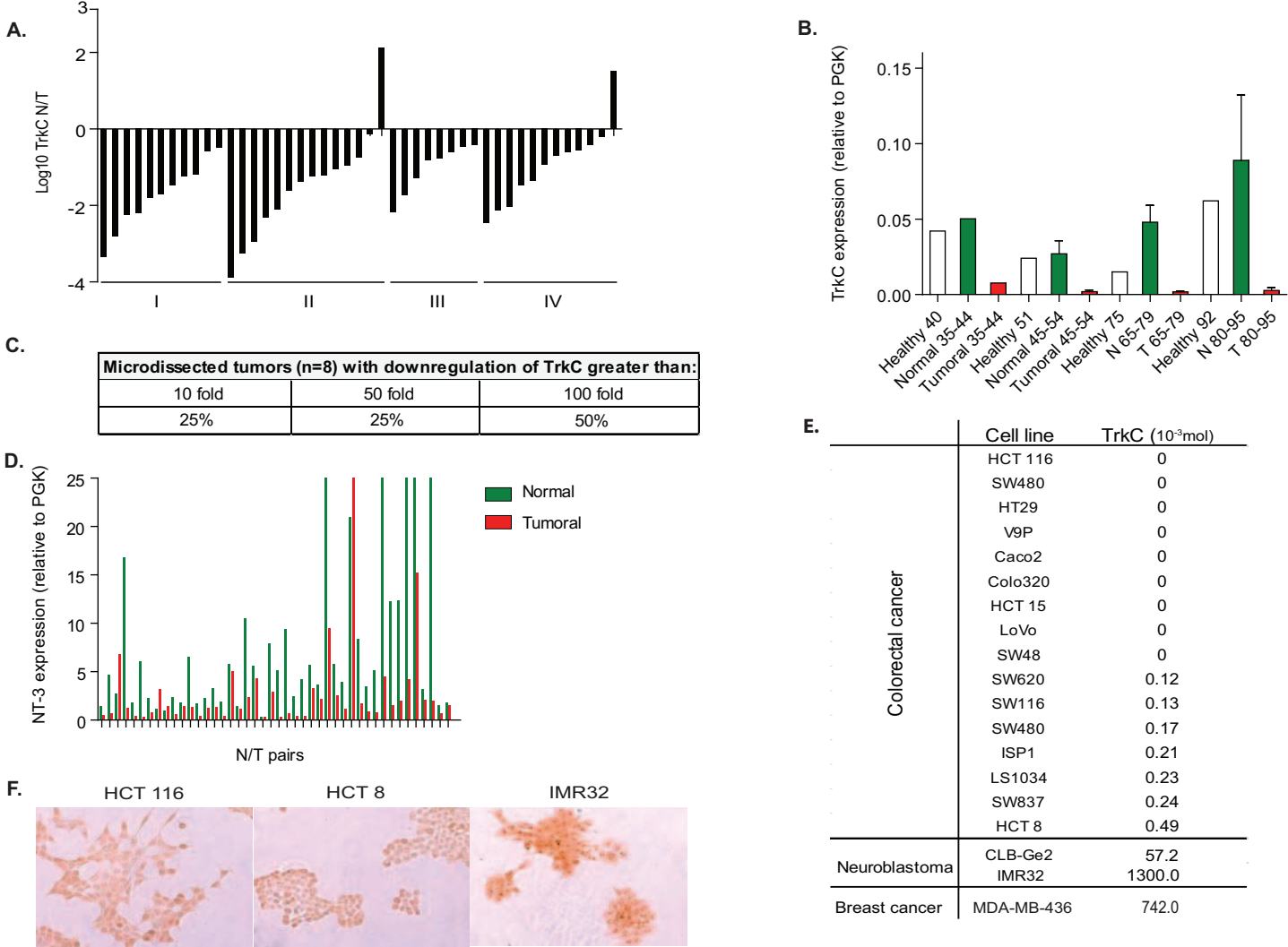
Representative pyrograms obtained for a pair of matched normal and tumoral samples. The sequence below the pyrograms indicates the sequentially added nucleotides. The yellow regions indicate the analyzed C/T sites; the proportion of C and T detected are provided as percentage values. This proportion corresponds to the ratio of methylated (C) and non methylated (T) DNA strands.

#### **Supplementary Figure 4. TrkC limits anchorage-independent growth and migration and induces apoptosis.**

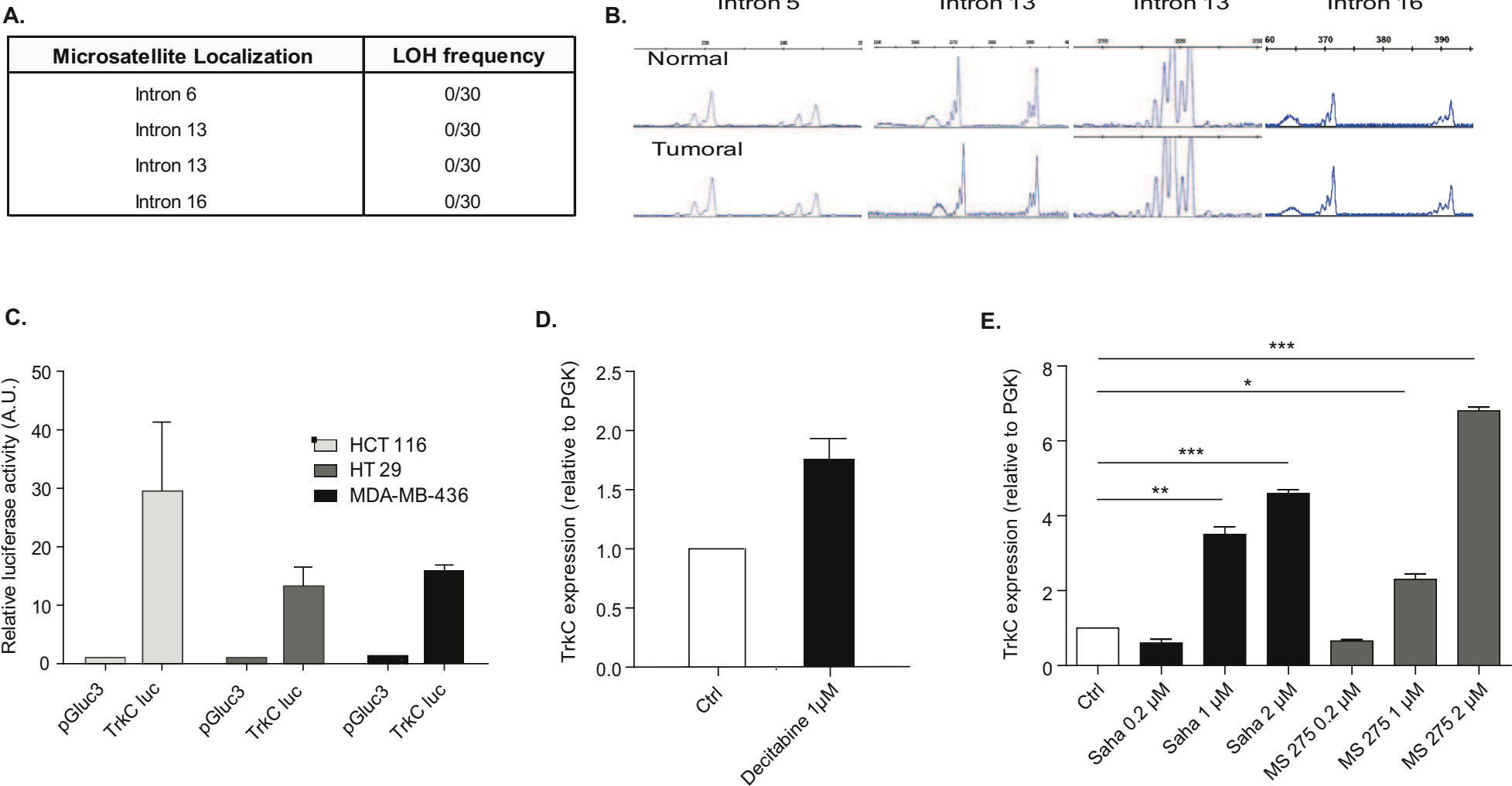
**(A)** Control, TrkC and TrkC KF over-expressing HCT 116 cells were grown in soft agar for two weeks. The number of colonies was counted in five random fields and the average number per field was calculated. Data represent mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$ , 2-sided Mann-Whitney test, compared with control. The size of colonies for each condition is shown. **(B)** Transwell assay was performed on HCT 116 cells

expressing control vector and TrkC with or without NT-3 or Z-VAD-fmk addition. The migration was stopped at 48 hours and the migrated cells were counted (right panel). Representative photographs are shown (left panel). **(C)** TrkC induces cell death in HCT 116 cells as shown by caspase-3 activity. **(D,E)** TrkC induces cell death in HCT 8 cells as shown by caspase-3 activity **(D)** and Toxilight assay **(E)**. Cell death is reversed by addition of NT-3 (10ng/ml). Data represent mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 2-sided Mann-Whitney test.

Supplementary Figure 1

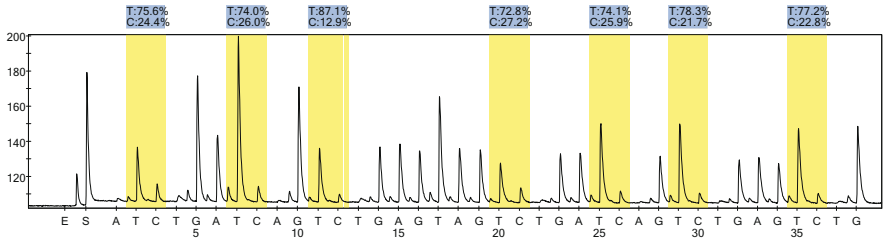


Supplementary Figure 2

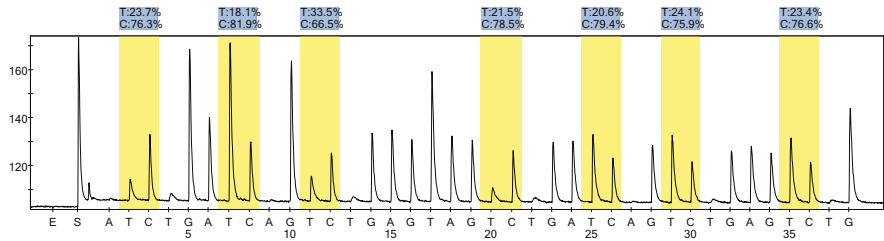


Supplementary Figure 3

Normal tissue

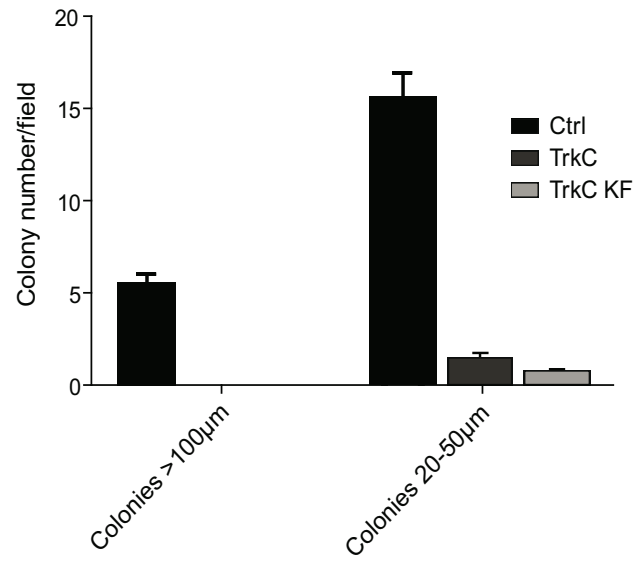


Tumoral tissue

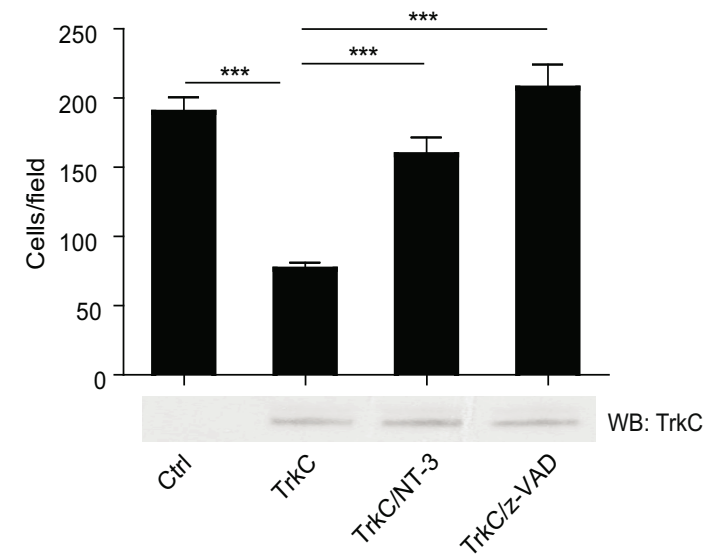
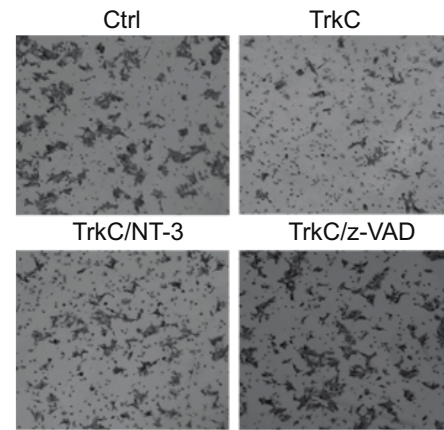


**Supplementary Figure 4**

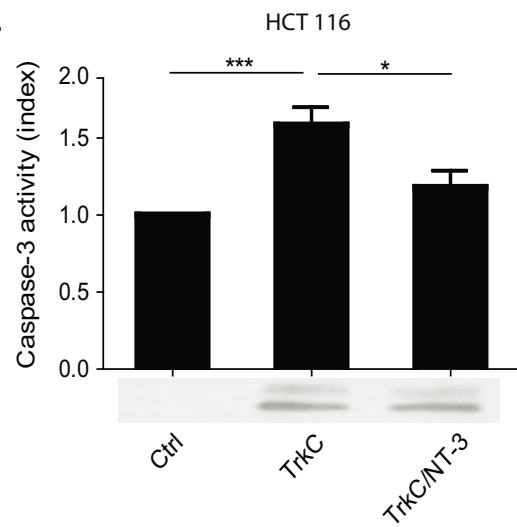
**A.**



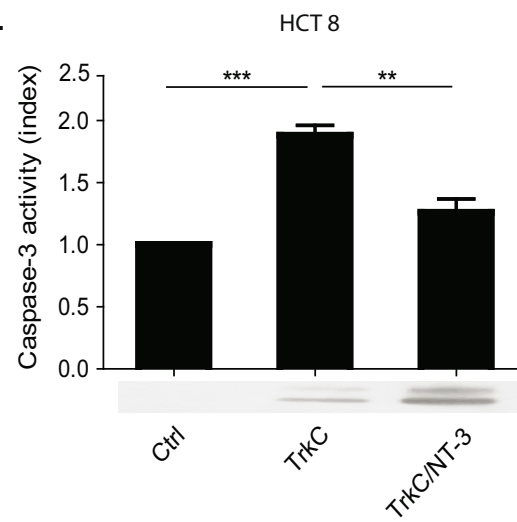
**B.**



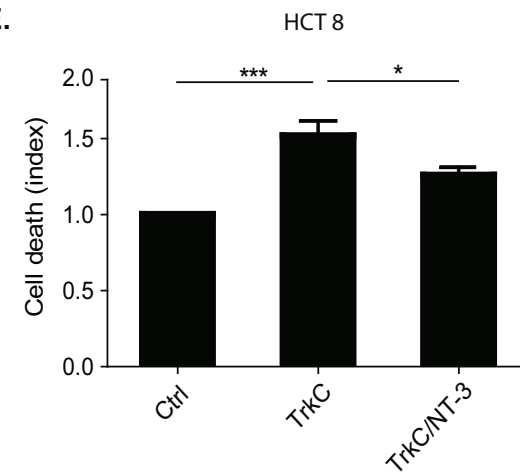
**C.**



**D.**



**E.**



## 2.5 Ongoing project: The characterization of TRKC-induced apoptosis

Our study on COBRA1 and the mitochondrial crosstalk is not exhaustive in the sense that many other possible interacting partners of TRKC KF are being investigated.

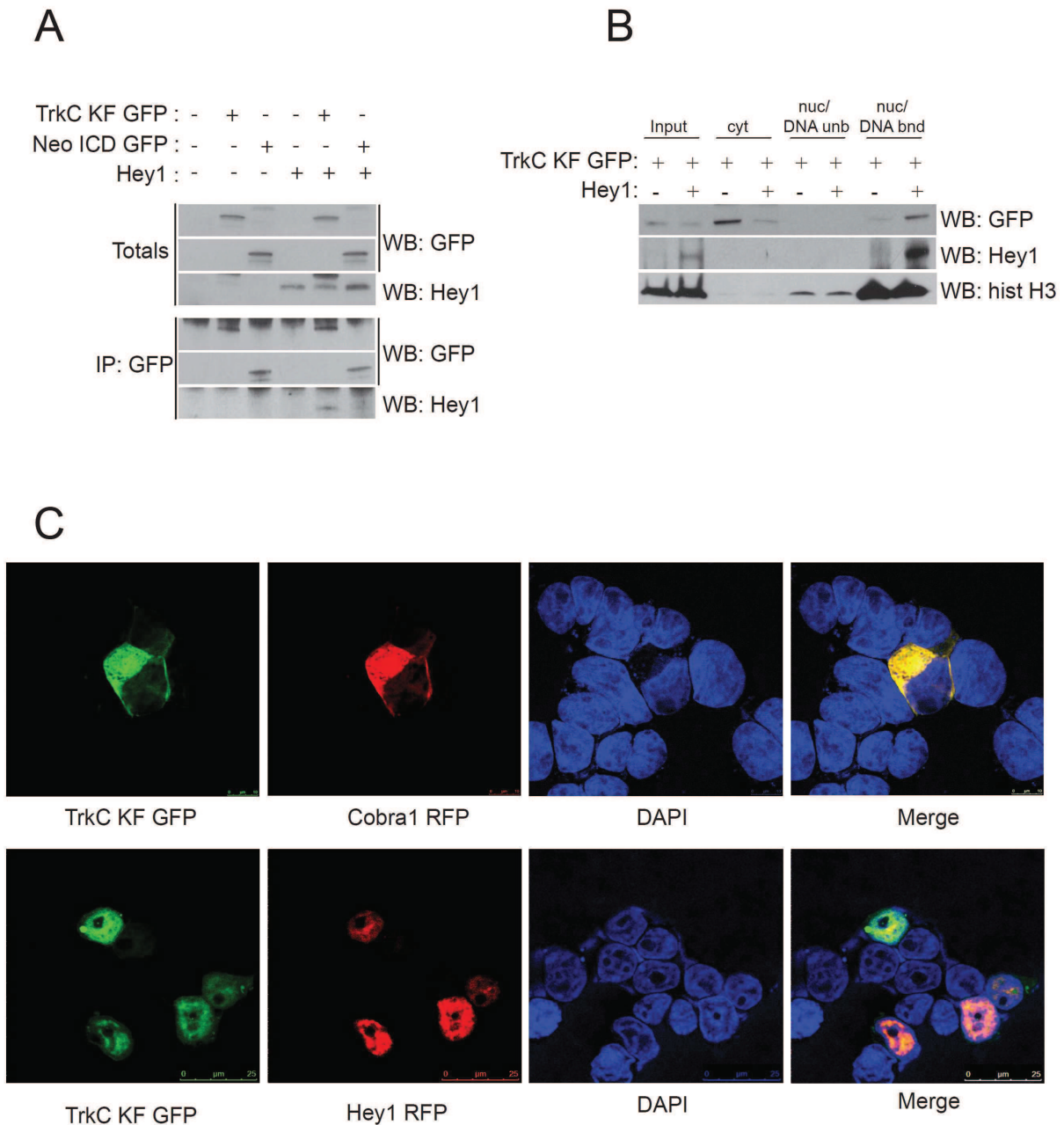
We are now confirming the interaction by CoIP between TRKC KF and WDR13 (a homologue of APAF-1, so possibly a caspase activating complex similar to the apoptosome might be involved in TRKC-induced cell death), importin  $\alpha 3$  (this could explain how a pool of TRKC KF is found in the nucleus) or BAF180 (a component of the SWI/SNF complex responsible for chromatin dynamics).

We are more advanced in studying HEY1, a transcriptional factor involved in NOTCH signaling, that was one of the hits in the two-hybrid screen. We have confirmed in HEK 293T cells that HEY1 interacts with TRKC KF while it does not with the intracellular domain of Neogenin, an unrelated receptor (Neo ICD) (Figure 5A). Interestingly, when co-transfected with HEY1, TRKC KF GFP was markedly localized in the nucleus, in the DNA bound fraction, as seen following HEK 293T fractionation into cytosolic (cyt), nuclear and DNA unbound (nuc/DNA unb) and nuclear and DNA bound (nuc/DNA bnd) fractions (Figure 5B). This was further confirmed by confocal microscopy; while it is accumulated in the cytosol following co-expression with COBRA1, TRKC KF GFP becomes nuclear when co-expressed with HEY1 (Figure 5C).

Even though it is also found in the nucleus, TRKC KF does not possess a gene transactivation activity (data not shown).

We hypothesize that HEY1 binds and accumulates TRKC KF in the nucleus in order to protect the cell against the mitochondrial apoptosis triggered by TRKC. However, this will be broadly analyzed in the Discussion section.

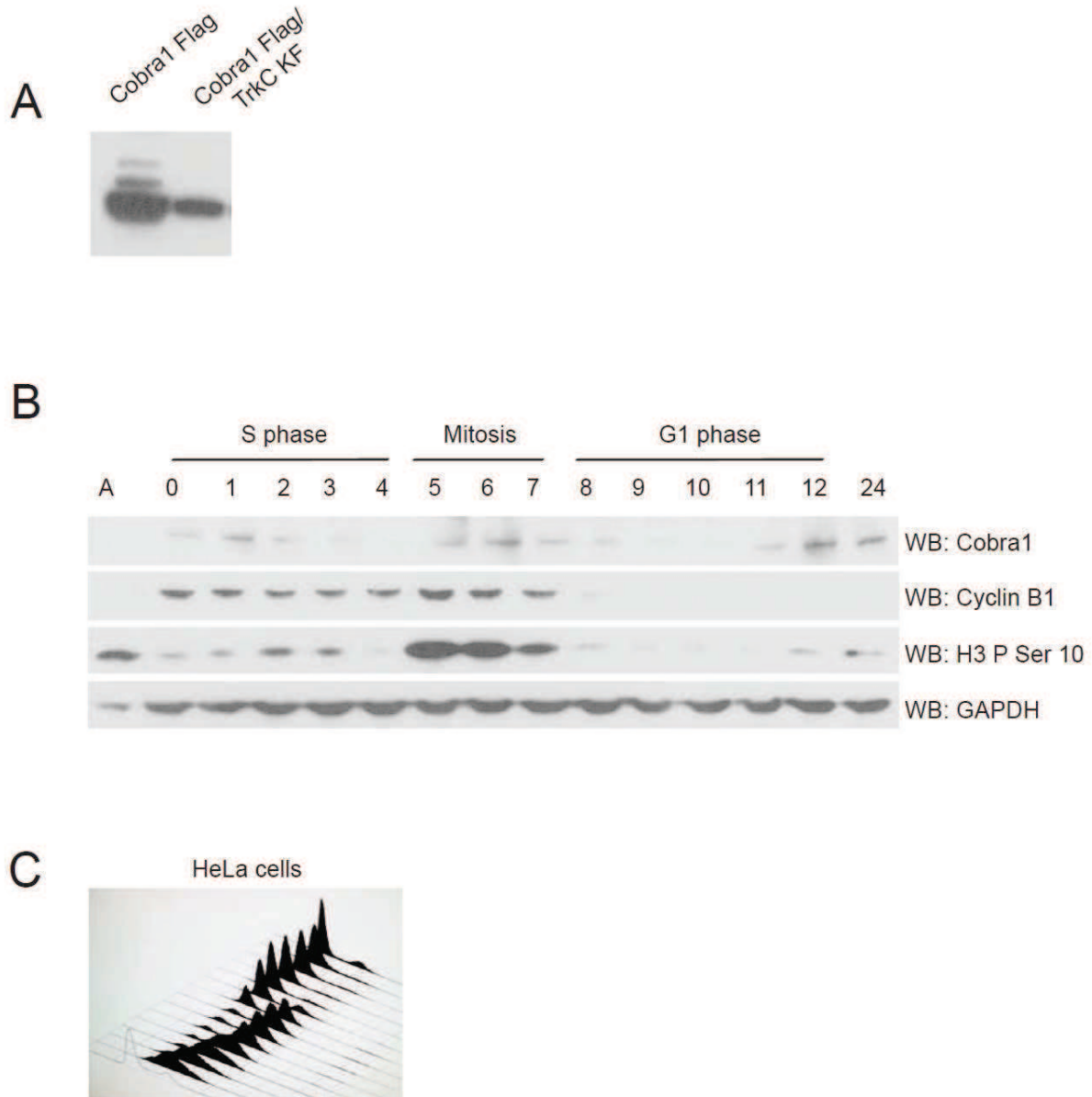




**Figure 5. TRKC KF GFP co-immunoprecipitates with HEY1 (A) and it is shuttled in the nucleus when the two proteins are ectopically co-expressed in HEK 293T cells (B, C).**

Another aspect which I am interested in is the post-transcriptional regulation of COBRA1. The starting point was the observation that when expressed alone, above COBRA1 in immunoblots there are several bands shaped as a ladder. This can be the result of poly-

ubiquitination, a post-transcriptional modification leading most likely to degradation by the proteasome (Thrower, Hoffman et al. 2000).



**Figure 6.** COBRA1 immunoblot presents ladder shaped-bands when expressed alone in HEK 293T cells (A). (B) HeLa cells were synchronized by double thymidine in G1/ S-phase and released for 24 hours. Immunoblots for COBRA1, CYCLIN B1, Phospho-serine 10 histone H3 and GAPDH are presented. (C) The cell cycle profile of the synchronized HeLa cells is depicted.

Since many proteins are degraded in cell cycle dependent manner, we have investigated if this is the case for COBRA1. To this end, HeLa cells were arrested at the G1/S boundary using a

double thymidine block followed by release into fresh media to allow cells to progress through cell cycle (Figure 6B, C). Phospho-serine 10 on histone H3 immunoblot was used to precisely mark the M (mitosis) phase while CYCLIN B1 is given as an example of protein degraded just before mitosis. As shown in figure 6B, COBRA1 protein levels are low in S-phase while higher in mitosis. This can already give us a hint about what ubiquitin-ligase complex may be involved in COBRA1 degradation. It is known that the ubiquitin-ligase SKP1-CUL1-F-box-protein complex (SCF) is mainly active in G1/S phase while the anaphase-promoting complex/cyclosome (APC/C) targets proteins for degradation in mitosis (Fasanaro, Capogrossi et al., 2010). Therefore we will focus for further studies on SCF.

### **3. Discussion and perspectives**

#### **3.1 Cell death induction by TRKC: initiation events**

##### **3.1.1 How is NT-3 making the difference between life and death?**

It became very common to stipulate that TRKC and, by extension all dependence receptor, do not induce apoptosis when bound by its ligand. But how exactly binding of the respective ligand impedes the apoptotic signal? Several hypotheses could be put forward.

First, it could be possible that ligand binding induce a certain conformation in the intracellular part of the receptor that does not allow the initial caspase cleavage. Along this line, it was shown that several dependence receptors such as DCC and UNC5H2 adapt a multimeric conformation when binding their ligands (Mille, Llambi et al. 2009). Furthermore, several tyrosine kinase receptors are known to dimerize when ligand-activated (Reichardt 2006), strengthening the possibility that while dimerization, the caspase cleavage site(s) are not accessible. Indeed, an *in silico* analysis of TRKC showed that dimerization may mask the aspartic residues targeted for caspase cleavage (J.G. Delcros, unpublished data). Similarly, receptor phosphorylation may have the same effect. This was reported for the dependence receptor MET, in which phosphorylation in Y1001 inhibits caspase cleavage at D1000 (Deheuninck, Goormachtigh et al. 2009).

Second, TRKC-induced apoptosis may be inhibited by the pro-survival Ras/MAPK and PI3K/AKT pathway that are activated following NT-3 binding. This view is supported by several evidences: when the PI3K/AKT pathway is turned on, it leads to BAD phosphorylation and consequent sequestration by the chaperone 14-3-3. Similarly, FOXO3A is inactivated and therefore it cannot activate the transcription of pro-apoptotic effectors such

as FASL, TRAIL, PUMA and BIM. Moreover, NT-3 equally activates the pro-survival NF- $\kappa$ B and CREB pathways (reviewed in Ichim, Tauszig-Delamasure et al., 2012).

### **3.1.2 Caspase activation: initiation or execution program?**

As mentioned before many DRs need caspase cleavage in order to initiate the apoptotic process. Additionally, it is thought that DRs amplify the caspase cleavage cascade by themselves or by recruiting a caspase-activating complex (the dependosome) as it is the case of the receptor PATCHED (for review see Thibert and Fombonne, 2010).

Then, how is it possible that caspase cleavage, believed to be a terminal event, is in fact an initiation event in the apoptotic cascade initiated by the DRs? One possible explanation is that pro-caspases are not completely inactive, having a weak proteolytic activity when brought in close proximity with substrates (Yang, Chang et al. 1998). Moreover, many substrates are cleaved by caspases even though this does not lead to cell death. These substrates include members of diverse protein families such as transcription factors, cytokines, kinases or polymerases (Lamkanfi, Festjens et al. 2007). Several mechanisms are carefully designed to restrict this cleavage and avoid the dismantling of the cell. These include post-transcriptional modifications and insulation of pro-caspases or even active caspases in different cell compartments (e.g. pro-caspase-3 in the mitochondria and pro-caspase-9 in the nucleus) (Launay, Hermine et al. 2005). In addition, the expression of endogenous inhibitors of caspases such as IAPs implicates an internal mechanism to prevent the propagation of active caspases (Salvesen and Duckett 2002). It seems therefore possible that DRs mediated apoptosis is in fact initiated by very localized receptor cleavage that will further amplify caspase activation leading to cell death.

There are more and more evidences suggesting that several DRs recruit a protein complex inducing their own caspase cleavage, followed by the release of an apoptotic domain that will amplify the cell death cascade. Preliminary results indicate that similar to PATCHED, unliganded TRKC is able to recruit the adaptor protein DRAL (Down-regulated in Rhabdomyosarcoma LIM-domain protein; also known as FLH2), one of the caspase recruitment (CARD)-domain containing protein NALP1 (NLR family, pyrin domain containing 1) and caspase-9. These proteins forming the so-called dependosome were shown to activate locally caspase-9 that will double-cleave TRKC in the intracellular domain leading to TRKC KF formation (Lionel Giraud and Gabriel Ichim, unpublished data).

Additionally, the truncated receptor itself could amplify the pro-apoptotic signal by fixing the ligand, as it is the case of MET (Deheuninck, Goormachtigh et al. 2009). One can imagine that truncated TRKC could amplify the apoptotic signal by this mean. However, further work is needed to support this hypothesis.

## **3.2 TRKC crosstalk with the mitochondria**

### **3.2.1 Mitochondria involvement: is TRKC alone?**

We have recently shown that TRKC KF, the fragment generated by caspase cleavage of TRKC at aspartic residues D495 and D641 is partially addressed at the mitochondria, where it binds and activates BAX. This leads to the release from the mitochondrial inter-membrane space of several toxic proteins such as cytochrome *c* and OMI. Cytochrome *c* forms eventually the apoptosome, activating caspase-9, which on its turn activates the executioner caspase-3.

It would not be the first report of a protein which gives rise by caspase cleavage to pro-apoptotic mitochondrial-targeted fragments. For instance, it was shown that BAD is cleaved

by caspase(s) in N-terminus to generate a 15-KDa truncated protein. This tBAD is detected exclusively in the mitochondrial fraction where it interacts preferentially and inhibits the pro-survival function of BCL-XL, leading to a more efficient MOMP (Condorelli, Salomoni et al. 2001). Similarly, following TRAIL treatment, MCL-1 is cleaved by caspases releasing a C-terminus fragment that will become highly apoptotic due to physical interaction and cooperation with tBID and BAK (Weng, Li et al. 2005). The tyrosine kinase receptor HER2 was also described to be cleaved by caspases, releasing two BAD-like fragments that localize at the mitochondria and induce cytochrome *c* release (Strohecker, Yehiely et al. 2008). Similar studies were done on HER4 (Naresh, Long et al. 2006), p53 (Sayan, Sayan et al. 2006), hepatitis c virus core protein (Mohd-Ismail, Deng et al. 2009), actin (Utsumi, Sakurai et al. 2003) or the tight junction protein MAGI-1 (Ivanova, Repnik et al. 2007).

There are several evidences that other dependence receptors triggered-cell death involve the intrinsic pathway. It is the case of the tyrosine kinase MET which is cleaved by caspases releasing a 40-KDa fragment (P40). P40 MET localizes at the mitochondria, interacts with BCL-2 and induces mitochondrial permeabilization leading to cytochrome *c* release (Jonathan Levebvre, unpublished data). Similar preliminary data concern also the dependence receptor PATCHED. Indeed, the ectopic expression of PATCHED in HEK 293T cells leads to a loss of mitochondrial transmembrane potential. Moreover, PATCHED-induced apoptosis is blocked by BCL-2 and seems to require the pore forming proteins BAK and/or BAX since no apoptosis induction is observed in BAK/BAX invalidated MEFs (Mathilde Bonnet, unpublished data).

Finally, there are few *in vivo* studies supporting the cross-talk between the dependence receptor TRKC and the mitochondrial apoptosis. First, the loss of TRKC-expressing proprioceptive neurons in NT-3 KO mice is rescued by the deletion of BAX (Patel, Kramer et al. 2003), the same fact being reported with NGF, the ligand of the dependence receptor



TRKA (Patel, Jackman et al. 2000). Moreover, BAX deficient one month-old mice have increased number of TRKC-positive neurons, suggesting that BAX could be involved in TRKC-induced apoptosis (Suzuki, Aoyama et al., 2010). Interestingly, BCL-XL KO mice present at E11.5 three times more neurons in the DRGs implying a protective role of BCL-XL against the programmed cell death occurring during developmental neuronal apoptosis (Urase, Momoi et al. 1999).

However, even if these studies may imply a role of the mitochondrial pathway in the cell death of TRKC-expressing neurons, there is certainly a large fraction of this apoptosis that is triggered by the well-described now neurotrophic factor withdrawal and therefore by the absence of the pro-survival signals initiated by neurotrophins. We think nevertheless that a part of this cell death is initiated by the unliganded TRKC. Additional evidences are needed to support this hypothesis and mainly the remaining questions could be answered by crossing the different knock-out mice described above with a knock-in mouse carrying a non-cleavable TRKC.

### **3.2.2 BAX activation – Where TRKC KF stands?**

Most of the cell death occurring in vertebrae involves the mitochondrial pathway of apoptosis that is controlled by the interplay between proteins of the BCL-2 family. When various stress factors unbalance the interactions between these proteins, the two pro-apoptotic BCL-2 effectors, BAX and BAK disrupt the integrity of the mitochondrial outer-membrane, resulting in the so-called MOMP (Walensky and Gavathiotis, 2011). This permeabilization appears to be in the shape of large pores of 25-100 nm in diameter that allow the diffusion of the IMS proteins (Schafer, Quispe et al. 2009). How exactly BAX and BAK cause MOMP is still a

matter of intense debate since there are little structural evidences about the pore-forming capacity of BAX and BAK. However, great efforts were done lately in this direction.

The activation of BAX and BAK is mainly triggered by the BH3-only proteins BID and BIM. The case of BID is the best characterized so far: first, active BID (truncated, tBID) associates with the mitochondrial membrane where it transiently binds and activates BAX; secondly, active BAX anchors itself in the outer mitochondrial membrane where it binds other BAX molecules and therefore forming the pores responsible for MOMP (Lovell, Billen et al. 2008). Of interest, other direct activators of BAX or BAK have been recently described. The receptor ERBB4/HE4 was shown to release by proteolytic processing a BH3-domain containing fragment that accumulates at the mitochondria and induces BAK activation (Naresh, Long et al. 2006). Similarly, endophilin B, involved in the dynamics of the outer mitochondrial membrane (Etxebarria, Terrones et al. 2009) and the transcription factor IRF-3 (Chattopadhyay, Marques et al., 2010) appear to activate BAX. The latter induces BAX activation independently of its transcriptional activity.

In this specific context, we are describing that TRKC KF, one of the proteolytic cleavage fragments of the dependence receptor TRKC is able to interact and activate BAX. TRKC KF does not possess a functional BH3 domain, however the interaction with BAX is not surprising since other proteins lacking BH3 domain(s), as it is the case of the cytosolic p53 or IRF-3, were previously described to activate BAX (Chipuk, Kuwana et al. 2004; Chattopadhyay, Marques et al., 2010). Even though we provide evidences of the physical interaction between TRKC KF and BAX we cannot yet shed light on the structural characteristics of the TRKC KF-BAX complex and how exactly BAX is activated. A first question we need to address is where in the cell this interaction takes place: is it in the cytoplasm or at the mitochondrial membrane? Both scenarios are possible since TRKC KF is found both in the cytosol and at the mitochondria. If it is in the cytosol, we can speculate that

TRKC KF may be involved in the recruitment of BAX at the mitochondria. However, if the interaction takes place at the mitochondria, then TRKC KF may have a role in the dimerization and pore-forming capacity of BAX. An experimental approach that may help us better characterize the localization and kinetics of TRKC KF-BAX interaction could be the use of the bimolecular fluorescence complementation (BiFC) technique. BiFC uses split fluorescent proteins that alone are not fluorescent but when fused to interacting proteins associate to form the fluorescent protein (Shyu, Liu et al. 2006). The technique was successfully used to visualize caspase-2 activation platform that occurs in the cytoplasm and not in the nucleus, as thought before (Bouchier-Hayes, Oberst et al. 2009).

Since after all, BAX is a toxic protein, the cells evolved different strategies to keep it under control. First, BAX was shown to be ubiquitinated by IBRD2, an IBR-type RING-finger E3 ubiquitin ligase that has been already implicated in p53-dependent apoptosis (Benard, Neutzner et al., 2010). This leads to a rapid proteasomal degradation of BAX. Intriguingly, it was shown that the turnover of BAX $\beta$ , the 24-kDa splice variant of BAX that is constitutively active and therefore more dangerous, is considerably higher than the turnover rate of the more abundantly expressed 21 kDa BAX $\alpha$  (Fu, Sukumaran et al. 2009). Moreover, it was demonstrated that phosphorylated BAX is easily bound by the peptidyl-proline isomerase PIN1 that will prevent the exposure of the pro-apoptotic N-terminal domain of BAX, as well its cleavage and mitochondrial targeting (Shen, Esnault et al. 2009).

In this light, it would be interesting to investigate if TRKC KF-BAX interaction affects also the stability of BAX. In this purpose we could do a cycloheximide chase that will exactly pinpoint the half-life of BAX in the absence or presence of TRKC KF. It would also be possible to verify by using MG-132 if the binding by TRKC KF impedes the proteasomal degradation of BAX.

Recently, an elegant study from the team of Richard Youle, demonstrated that in healthy cells BAX translocates constantly to the mitochondria, where BCL-XL retrotranslocates it back into the cytoplasm. Therefore the pool of mitochondrial BAX that is susceptible for activation is constantly kept under control (Edlich, Banerjee et al., 2010). One would be intrigued to investigate if TRKC KF prevents this retrotranslocation that would result in BAX unrestricted access to the mitochondria, activation and ultimately apoptosis.

### **3.2.3 TRKC and the mitochondrial dynamics**

The mitochondria are far from being static organelles as previously believed. In fact, they present as a highly dynamic network resulting from multiple fusion and fission events. The fusion process is believed to be important for maintaining the integrity of the mitochondrial genome and exchanging mitochondrial content, whereas fission is required for the turnover of damage mitochondria via mitophagy. Any imbalance between the two processes results in multiple consequence such as neuropathy, optic atrophy or neonatal lethality (Huang and Frohman, 2012).

The proteins regulating mitochondrial dynamics include the fission mediators DRP1 (Dnm1 in yeast) and FIS1, as well as the fusion mediators MFN1 and 2 (Fzo1 in yeast) and OPA1 (Mgm1 in yeast) (Suen, Norris et al. 2008). It was equally shown that members of the BCL-2 protein family can affect the mitochondrial dynamics. More specifically, several studies bring evidences that BAX and BAK but also BCL-XL and BCL-w favor either mitochondrial fusion or fission (Karbowski, Norris et al. 2006) (Berman, Chen et al. 2009) (Liu and Shio 2008). In addition, an unexpected crosstalk between BAX activation and DRP1 was described: the fission induced by DRP1 provides the appropriate membrane curvature and lipid composition for BAX activation (Montessuit, Somasekharan et al., 2010). In this context we could investigate if TRKC-induced apoptosis is in part due to a direct effect on mitochondrial fusion

and/or fission. Importantly, we have noticed that some cells in which TRKC KF was ectopically expressed present fragmented mitochondria indicating that probably mitochondrial fission/fusion was affected.

Several methods can be used to assess fusion/fission events. The classical mitochondrial fusion assay is using two populations of cells in which the mitochondria were separately labeled with two different fluorescent proteins. These two populations are mixed and cellular fusion is induced with polyethylene glycol (PEG). The subsequent mitochondrial fusion is therefore assayed by scoring the extent of coincidence between the two fluorescent proteins (Huang and Frohman, 2012). The mitochondrial dynamics can be also studied using the newly described photoactivatable GFP (PA-GFP) that can be activated by photoconversion within an individual mitochondrion. A fusion event is characterized by the transfer of activated PA-GFP molecules to unlabeled mitochondria while fission is followed by the loss of PA-GFP continuity (Molina and Shirihai 2009).

### **3.3 New pro-apoptotic interacting partners**

#### **3.3.1 The “dependosome” – a caspase-9 activating platform for TRKC?**

Even though we have rounded up the story of COBRA1 involvement in TRKC-induced cell death via the mitochondrial pathway, we are still interested in a better characterization of this signaling cascade.

One of the questions we are trying to address at the moment is if the unbound TRKC recruits the components of the “dependosome”, similar to the dependence receptor PATCHED. This could explain how caspase-9 is locally activated in order to double-cleave the intracellular part of TRKC, leading to the generation of the pro-apoptotic fragment TRKC KF. More

specifically, it was demonstrated that, in the absence of the ligand SHH, PATCHED recruits a protein complex formed of DRAL, the CARD domain containing protein NALP1 and caspase-9. The dependosome is therefore a platform for activating the initiator caspase-9 that will further control the downstream activation of effector caspases. It is commonly known that initiator caspases are typically activated following assembly of multi-protein complexes. The best characterized caspase-activating complexes in mammals include the apoptosome (APAF-1, cytochrome *c*, pro-caspase-9), the DISC (FAS, FADD, caspase-8), the PIDDosome (PIDD, RAIDD, caspase-2) and the inflammasome (NALP1, ASC, caspase-1 and -5) (Park, 2012). Our preliminary data show that following NT-3 deprivation, TRKC recruits all the components of the dependosome that is responsible for the activation of caspase-9 and -3. The silencing of either DRAL or NALP1 impedes TRKC-induced apoptosis both *in vitro* and *in vivo*, in a model of chick embryo tumorigenesis (Lionel Giraud and Gabriel Ichim, unpublished data).

Of interest, NALP1 has been reported to induce apoptosis, either through a direct association with caspase-2 and -9 or indirectly, through an interaction with APAF-1 and subsequent enhancement of apoptosome function (Tschopp, Martinon et al. 2003). Therefore it would be interesting to test if APAF-1 is part of the dependosome complex recruited by unbound TRKC. Moreover, a preliminary study described the interaction between PATCHED and the ubiquitin ligase X-linked inhibitor of apoptosis (XIAP), which could be therefore another component of the dependosome. Since XIAP ubiquitinates caspase-9 leading to its degradation, it would be challenging to test whether XIAP is keeping under control the activated caspase-9 in the dependosome ensuring only local caspase activation responsible for receptor cleavage.

Similarly, it would be interesting to unravel the stoichiometry of the dependosome since the large size of it (1000-2000 kDa) suggests the existence of other components that may be involved in TRKC-induced apoptosis.

### 3.3.2 Other possible interacting partners

In the idea of indentifying pro-apoptotic interacting proteins that could be relevant for the cell death induced by the unbound TRKC, we have performed a yeast two-hybrid screen using TRKC KF as bait against a mouse embryonic brain cDNA library. Many interesting putative interacting partners were found and some of them are discussed here.

- **HEY1** is a transcription factor belonging to the vertebrae basic-helix-loop-helix-Orange (bHLH-O) superfamily of transcriptional repressors, together with HEY2 and HEYL (Davis and Turner 2001). NOTCH pathway was shown to increase HEY1 expression, leading to transcriptional inhibition of downstream targets. Therefore, HEY1 appears as critical effector of NOTCH, being involved in cardiac and vascular development (Fischer, Schumacher et al. 2004), neurogenesis (Sakamoto, Hirata et al. 2003) and bone development (Zamurovic, Cappellen et al. 2004). Intriguingly, a genome-wide analysis showed that HEY1 is a positive regulator of the tumor suppressor protein p53. Ectopically expressed HEY1 activates p53 and induces apoptosis in vitro through inhibition of MDM2 and post-translational stabilization of p53 (Huang, Raya et al. 2004). An interesting study that might link HEY1 to the pro-apoptotic pathway of TRKC shows an increased number of TRKC expression neurons in the DRGs, following HEY1 invalidation (Mukhopadhyay, Jarrett et al. 2009).

The co-immunoprecipitation and the sub-cellular fractionation experiments clearly show an interaction between TRKC KF and HEY1, with a translocation of TRKC KF

in the nucleus. However we are not able at the moment to demonstrate if HEY1 is a pro- or anti-apoptotic partner of TRKC. Even though TRKC KF does not have gene transactivation activity, we cannot exclude an effect on the transcriptional suppressor activity of HEY1. One can imagine that TRKC KF may enhance the positive regulation of HEY1 on p53, leading to increased cell death. Other pro-apoptotic effectors may also be activated through a similar mechanism. An unbiased answer to this problem can be given by a genome-wide expression analysis.

We can also hypothesize that HEY1 is protective against a dangerous cleavage fragment such as TRKC KF by capturing it in the nucleus. This would be not the first example of nuclear sequestration of dangerous proteins. For example, the oncoprotein c-MYC is sequestered in the nucleoli where this central regulator of cell proliferation will be degraded via the proteasome (Arabi, Rustum et al. 2003). Similarly, the human herpes virus 8 (HHV-8) blocks the pro-apoptotic BIM in the nucleus as a mean of inactivation (Choi and Nicholas, 2010).

- **Importin-alpha3 (KPNA4)** - The alpha and beta importins are responsible for the nuclear import of hundreds of proteins. Importin-alpha3 binds the classical nuclear localization signal (NLS)-containing proteins and links them to importin beta that will ferry the complex through the nuclear-pore complex (NPC). The importins will then be recycled back into the cytoplasm by the exportins (Goldfarb, Corbett et al. 2004). Among the targets of importin-alpha3 are the hypoxia-inducible factors and p53 (Depping, Steinhoff et al. 2008; Marchenko, Hanel et al., 2010). Since TRKC KF does not have a *bona fide* NLS, it would be possible that TRKC KF-HEY1 complex (Hey1 having a NLS) may be targeted in the nucleus via the importins. The invalidation of the endogenous importin-alpha3 would give us an indication about the involvement of this putative partner in TRKC-induced cell death.



- **BAF180 (PBRM1)** is a subunit of PBAF (polybromo/Brg1-associated factor), a chromatin remodelling complex which is critical in mediating the process of transcription, replication, DNA repair and recombination (Vignali, Hassan et al. 2000). BAF180 was recently found mutated in a very high percentage of renal cell carcinoma (Varela, Tarpey et al., 2012) and it was also indentified in a screen looking for tumor suppressor genes in breast cancer (Xia, Nagase et al. 2008). BAF180 is also on the top list of genes important for prevention of faulty senescence since when disrupted it was found to promote proliferation (Burrows, Smogorzewska et al., 2012). The mechanism through which BAF180 exerts its tumor suppression function seems to involve p21 and p53 that are both regulated by BAF180 (Xia, Nagase et al. 2008; Burrows, Smogorzewska et al., 2012). Our preliminary data is limited to showing the interaction between TRKC KF and BAF180 in the nuclear fraction and also we have tried to assess the effect of TRKC KF on the transcriptional regulation of p21. We can speculate that a TRKC KF-BAF180 complex can have an enhanced effect on the transcriptional regulation of p53 which can then activate critical inducers of the intrinsic pathway such as BAX, NOXA, PUMA and APAF-1 (Yoshida and Miki, 2010).
- **WDR13** is a newly described member of the WD-repeat protein family (Singh, Suresh et al. 2003). The WD-repeats contain a conserved core of approximately 40 amino acids that usually end with tryptophan-aspartic acid (WD) and form a circularized  $\beta$  propeller structure (Neer, Schmidt et al. 1994). The importance of these proteins is better illustrated by their essential roles in very diverse biological function ranging from signal transduction, transcription regulation, to apoptosis. APAF-1 is a member of this family, having 12 WD repeats in the C-terminal region responsible for protein-protein interaction (Zou, Henzel et al. 1997). *In vitro* binding assays showed that WD-

repeats are responsible for binding cytochrome *c*, formation of APAF-1 oligomers and binding to pro-caspase-9 (Li and Roberts 2001). In regards to our study, we can suppose that unbound TRKC is able to recruit WDR13 molecules that self-associate like APAF-1 to form a caspase-activating complex required for the initiation of TRKC-induced cell death. Another working hypothesis is that WDR13 negatively regulates IAPs (inhibitor of apoptosis) since WD-repeat proteins can bind IAPs (Pascal Meier, personal communication).

- **FASN (Fatty Acid Synthase)** is a 270 kDa cytosolic dimeric enzyme that catalyses the terminal steps in the *de novo* biogenesis of fatty acids (Liu, Liu et al., 2010). Several lipids some of which are issued from FASN-mediated reactions such as phosphatidic acid, diacylglycerol, cardiolipin and cholesterol have been discovered to be important for the mitochondrial membrane morphodynamics (Huang and Frohman, 2012). Interestingly, cholesterol was been shown to be enriched in outer membrane contact sites where it may be involved in the stabilization of curved membrane microdomains while cardiolipin is required for DRP1-mediated Bax activation (Furt and Moreau 2009; Montessuit, Somasekharan et al., 2010). Since cholesterol content on the outer mitochondrial membrane was shown to be crucial for the ability of BAX to activate and induce MOMP, we may suspect that TRKC may influence the activity of FASN leading to a modulation of mitochondrial cholesterol content.

FASN is overexpressed in a multitude of human cancers, including cutaneous melanoma, in which its levels of expression are associated with tumor invasion and poor prognosis (Menendez and Lupu 2007). Interestingly, the pharmacological inhibition of FASN in melanoma cells results in the activation of the intrinsic pathway of apoptosis through the release of cytochrome *c*, and activation of caspase-9 and -3. Unpublished observations associated also FASN inhibition with changes in the

mitochondria free fatty acid composition and probably mitochondrial membrane fluidity (Zecchin, Rossato et al., 2010). In this context, maybe we can broaden our view of how TRKC can induce apoptosis. The mechanism is most probably more complex than the one we propose at the moment, with a mitochondrial targeting of TRKC KF, MOMP and activation of effector caspases. The signaling cascade may therefore involve inhibition of FASN, followed by modification of lipid composition in the mitochondrial outer membrane and facilitation of BAX activation.

### **3.4 Therapeutic perspectives**

#### **3.4.1 NT-3 up-regulation, a mechanism to inhibit TRKC-induced cell death?**

The expression of TRKC and its ligand NT-3 was screened in various types of cancers, however most of them focused on the presence of TRKC. The reason is that TRKC is a RTK and therefore the classical view is that it can function as an oncogene driving tumorigenesis when abnormally activated. This was generally valid in most of the cancers, but the role of TRKC was less clear in cancers where its expression has been shown to be a marker of good prognosis (Svensson, Ryden et al. 1997; Nakagawara 2001). On its turn, NT-3 has been considered only the ligand of an oncogenic TRKC that allows its constitutive activation.

Nevertheless, if we reconsider these studies through the perspective of DR theory we can argue that NT-3 up-regulation results in cell survival by activating RTK-induced positive signaling but also by inhibiting TRKC-induced cell death. We argue indeed that NT-3 up-regulation may confer a selective advantage to tumor cells since it inhibits TRKC mediated cell death and we provide evidence for this in the case of neuroblastoma (Bouzas-Rodriguez, Cabrera et al., 2010). It has been already proposed that TRKC expression is associated with favorable clinical outcome due to its ability to induce apoptosis. Pomeroy and colleagues

demonstrated that TRKC expression in medulloblastoma primary cultures is highly correlated with TRKC apoptotic response. Moreover, the ectopic expression of TRKC in DAOY medulloblastoma cells inhibits tumor growth of intracerebral xenografts in nude mice (Kim, Sutton et al. 1999). Even though these results have been interpreted under the scheme of TRKC being activated by NT-3, we think that the dependence receptor function of TRKC could provide an alternative explanation.

### **3.4.2 The mechanism(s) behind NT-3 up-regulation and loss of TRKC expression**

The highlight of our recent study on neuroblastoma is that a large subset of aggressive neuroblastoma tumors has an up-regulation of NT-3 which facilitates tumor growth and metastasis (Bouzas-Rodriguez, Cabrera et al., 2010). This was equally observed in breast and kidney cancer (Anne-Laure Genevois, unpublished data). Given these data, we investigated the mechanism(s) controlling the up-regulation of NT-3. Our preliminary data point towards an epigenetic regulation of NT-3. Indeed, when comparing the NT-3 positive (CLB-Ge2) and NT-3 negative (IMR32) cell lines we noticed that methylation inhibition using 5-azacytidine, an inhibitor of DNA methyltransferases, restored NT-3 expression in IMR-32 (Bouzas-Rodriguez, Cabrera et al., 2010). Therefore, NT-3 up-regulation could be due to promoter hypomethylation. Along this line, several studies emphasized the role of epigenetic modifications in gene regulation, with a special attention given to promoter hypermethylation. By comparison, the global DNA hypomethylation observed in cancer was given less attention. The role of cancer-linked hypermethylation is clearly due to its consequent transcriptional silencing of tumor suppressors. On the other hand, the biological significance of DNA hypomethylation is less understood (Ehrlich 2002). Several cancer-associated genes were found to be hypomethylated resulting in aberrant gene expression. For example, the promoter of HOX11 oncogene was reported to be hypomethylated in T-cell lymphoblastic leukemia

resulting in gene expression up-regulation (Watt, Kumar et al. 2000). Similarly, the tight junction protein claudin-4 is aberrantly up-regulated in gastric cancer (Kwon, Kim et al., 2011).

The up-regulation of NT-3 in cancers can be also due to transcription factors miss-regulation. Using several transcription factors binding site prediction soft-wares (<http://www.phylofoot.org/consite/>; <http://www.cbrc.jp/htbin/nph-tfsearch>) we found putative binding sites for ZEB-2, NF- $\kappa$ B and HSF1 (Heat Shock Factor 1) in the promoter of NT-3.

- **ZEB-2** is one of the transcription factors involved in orchestration of the epithelial-mesenchymal transition (EMT), a key process both in embryonic development and cancer pathogenesis. EMT was described to give rise to cancer cells with stem cell-like characteristics that have enhanced capacity of invading surrounding tissue and are resistant to classical chemotherapy (Polyak and Weinberg 2009). Claudin-low tumors, an intrinsic subtype of breast cancer tumors, were shown to be enriched in EMT and cancer stem cell properties (Prat and Perou, 2011). Interestingly, we found an up-regulation of NT-3 in MDA-MB-231, MDA-MB-436 and Hs578T that are all claudin-low breast cancer cell lines. Taking this together, we can suspect a miss-regulation of NT-3 transcription in claudin-low breast cancer tumors due to ZEB-2 and probably other EMT-related transcriptional factors such as SNAI1, SLUG or TWIST1.
- The transcription factor **NF- $\kappa$ B** was recently associated with an increase expression of its transcriptional target netrin-1 in a fraction of colorectal tumors issued from patients affected by inflammatory bowel disease. It was therefore proposed that induction of netrin-1 expression via NF- $\kappa$ B could affect colorectal tumor progression (Paradisi and Mehlen, 2010) by blocking the cell death induced by DCC and UNC5H dependence receptors. A similar mechanism may apply for NT-3, however preliminary

experiments must be performed, especially in correlating the inflammation markers with NT-3 expression in breast cancer samples.

- The idea that **HSF1** could regulate the expression of NT-3 came from a genome-wide study which identified NT-3 among the genes regulated directly or indirectly by HSF1 (Page, Sikder et al. 2006). HSF1 is the master transcriptional regulator of heat shock proteins (HSPs) that are overly expressed by the cell to cope with proteotoxic stressors such as heat shock, hypoxia, free radicals or ATP depletion. HSPs act by promoting the refolding of miss-structured proteins or by diverting them to the proteasome when the protein miss-folding exceeds a certain threshold (Whitesell and Lindquist 2005). Intriguingly, there is an information gap about the role of HSF1 in cancer even though it is generally accepted that HSPs level increases in a wide range of tumor types. Given the fact that cancer cells are challenged by stressful insults, HSF1 can stimulate oncogenesis by facilitating cellular adaptation to the malignant lifestyle. In our opinion, this adaptation can also be achieved by transcriptional up-regulation of NT-3 that will promote cancer cell survival through the tyrosine kinase function of TRKC and moreover protect from the cell death induced by the dependence receptor function of TRKC.

The inactivation of TRKC can be also considered as an oncogenic strategy employed by tumor cells to evade TRKC apoptosis. We have recently submitted an article describing exactly this oncogenic mechanism (Genevois et al., under revision, PNAS). Indeed, we showed that TRKC is a conditional tumor suppressor in colorectal malignancies that is silenced due to promoter hypermethylation. As mentioned above, epigenetic event has been mostly associated with gene silencing and more particularly with the transcriptional blockage of several tumor suppressor genes. In colorectal cancer, aberrant methylation profile was

described for several tumor suppressor genes such as RB, APC, MGMT, RASSF2A, CDKN2A/P16, P14<sup>ARF</sup> or ECAD that were associated with tumor initiation, progression or invasiveness (Grady and Carethers 2008; Lee, Lee et al. 2009; Migheli and Migliore, 2010). In addition, our team also demonstrated that promoter hypermethylation is responsible for the silencing of the dependence receptor UNC5H in colon cancer (Bernet, Mazelin et al. 2007). Therefore it seems that epigenetic silencing is a general mechanism used by colorectal malignancies to down-regulate the apoptotic inducers TRKC and UNC5H.

### **3.4.3 Perspectives for TRKC/NT-3 targeted therapies**

The modern cancer therapy left behind the extensive use of nonspecific cytotoxic agents and moved toward rationally designed drugs that inhibit well-defined targets in certain types of cells.

The strategy of targeting TRKC/NT-3 interaction to re-induce apoptosis should be in fact theoretically considered a three-sided approach: first, an inhibition of TRKC/NT-3 interaction in cancers in which NT-3 is up-regulated; second, a complementary mitochondrial cell death-based therapy can be considered knowing that TRKC kills through the intrinsic pathway of apoptosis; third, epigenetic drugs (epidrugs) can be mixed in the attempt of re-expressing TRKC in cancers in which the expression of TRKC is lost or strongly diminished due to promoter hypermethylation. These three strategies will be shortly presented in the following paragraphs.

**Targeting TRKC/NT-3 interaction.** Since the first description that TRK tyrosine kinase receptors are involved in oncogenesis, therapeutic molecules have been designed to inhibit TRKs' kinase activity. CEP-701, CEP-2563 and CEP-751 (lestaurtinib) are tyrosine kinase inhibitors structurally related to staurosporine that compete with TRKs' ATP binding site. They have reached Phase I, II and III (CEP-701) clinical trials and their efficacy was

established in various cancers. However, these inhibitors must overcome two major drawbacks: first, since the kinase domains of TRKA, B and C share a high degree of homology, these inhibitors are not able to target a specific TRK receptor; second, mutations occurring on the kinase domain often render cancer cells resistant to these inhibitors (Tauszig-Delamasure and Bouzas-Rodriguez, 2011).

Our study on neuroblastoma proposed the alternative of developing interfering agents for TRKC/NT-3 binding that could be used in synergy with the already available TRK inhibitors. The aim of our future studies is to specifically target the binding between TRKC and NT-3. Considering the low homology between the extracellular domains of TRK receptors, the interfering agent will gain in specificity if it targets the extracellular domain of TRKC. The proof of concept was done using a polyclonal antibody blocking TRKC/NT-3 interaction. D. Rognan (Strasbourg) proceeded to a virtual screening of small molecules interfering with NT-3/TRKC binding. We have tested 40 first hits and have selected a promising molecule. This molecule is able to trigger specifically TRKC apoptosis in NT-3 overexpressing tumoral cells. Various chemical modifications will be required to ameliorate its pharmacokinetics properties and prevent this molecule from crossing the haematoencephalic barrier. Since NT-3 plays a critical role in cardiac development and it is present in the adult vascular smooth muscle (Hiltunen, Arumae et al. 1996; Donovan, Miranda et al. 1995), the potential off-target effects must be seriously considered.

**Mitochondria-based cancer therapy.** The link between tumorigenesis and impaired apoptosis became obvious when the anti-apoptotic protein BCL-2 that was described as an oncogene more than 20 years ago was found over-expressed in 90% of human follicular lymphoma (Adams and Cory 2007).

Since then several apoptotic regulators became therapeutic targets. However, since BCL-2 family members are intracellular proteins with no intrinsic catalytic function, their targeting



with antibodies or blocking small molecules was a challenging task. One of the first attempts was the use of antisense oligonucleotides (ASO)-based strategies that would interfere with mRNA expression (Kim, Emi et al. 2007). Oblimersen is one of the ASOs that is currently used in 45 clinical trials for the treatment of refractory CLL (chronic lymphoid leukemia), multiple myeloma and melanoma (source: Clinicaltrials.gov). Nevertheless, the most efficient drugs at the moment are the non-peptidic “BH-3 mimetics”, drugs that are mimicking the function of the pro-apoptotic BH3-only proteins. The most studied and promising BH3-mimetic to date is ABT-737 developed by Abbott. ABT-737 is a cell permeating synthetic BH3 mimetic that binds and inactivates BCL-2, BCL-XL and BCL-w but only weakly MCL-1 (Labi, Grespi et al. 2008). Concerning its *modus operandi*, ABT-737 acts as a “sensitizer” BH3-only protein that requires the presence of “direct activator” such as BAX and BAK to trigger cytochrome *c* release (Stauffer 2007). ABT-263 is a next generation BCL-2 family inhibitor already proved its efficacy on lymphoma, CLL and small cell lung cancer (SCLC) (source: clinicaltrials.gov).

The major drawback of ABT drugs is that they are not efficient on cancers displaying high levels of the BCL-2 pro-survival homolog MCL-1. Interestingly, Maurer and colleagues showed that growth factors are closely linked with the stability of MCL-1: following growth factors withdrawal, AKT levels are low releasing GSK-3 from an inhibitory phosphorylation loop; GSK-3 is therefore able to phosphorylate MCL-1 that will be then rapidly degraded (Maurer, Charvet et al. 2006). Very preliminary results indicate that NT-3 binding to TRKC stabilizes MCL-1, probably due to the same inhibition of GSK-3 (Gabriel Ichim, preliminary data). In this context, by combining a TRKC/NT-3 blocking antibody with a BH-3 mimetic we can probably achieve an enhanced apoptotic response: the lack of the trophic factor NT-3 results in diminished MCL-1 levels, the BH3-mimetic can achieve its inhibition on the other

BCL-2 family members and the unbound TRKC will trigger more easily the intrinsic apoptosis.

**The epidrugs.** Epigenetic mechanisms have long been known to be involved in oncogenesis, beginning with the initial observations that levels of DNA methylation were dramatically altered in most cancers. Taking advantage of the fact that epigenetic modifications are reversible, several inhibitors of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) were developed and proved their efficacy in cancer treatment (Jones and Baylin 2007). We can imagine the combined use of these inhibitors with the purpose of re-expressing the conditional tumor suppressor TRKC in cancers in which promoter hypermethylation is causing TRKC transcriptional silencing. Because drugs-mediated modulation of the epigenome lacks specificity, epidrugs can have side-effects such as oncogenic activation or stem cells reprogramming. Therefore, identification of inhibitors that are truly target-selective for their epigenetic protein will also help to tease apart the balance between efficacy and safety.

**To conclude, my thesis brought me closer to understanding the molecular mechanisms of TRKC-induced apoptosis. We have clearly established that TRKC kills through the mitochondrial pathway, with the caspase cleavage fragment TRKC KF able to be recruited at the mitochondria where it binds and activate BAX. The newly identified pro-apoptotic partner COBRA1 is responsible for the mitochondrial shuttling of TRKC KF.**

**Regarding the involvement of TRKC in oncogenesis, I took part in the study showing that TRKC acts as a conditional tumor suppressor both in neuroblastoma and colon cancer.**

## 4. Annexes

### 4.1. Annex 1: Neurotrophins and cell death (Review article)

In this review we discussed a more discrete side of neurotrophins and their receptors, meaning the cell death they are able to induce.

This apoptosis can be the result of a “default” program, meaning that neurons lacking neurotrophic support are scheduled to die without activation of any neurotrophic receptors. It is the case of the neuronal apoptosis induced by the withdrawal of NGF in TRKA-expressing sympathetic neurons. In this scenario, the lack of NGF results in expression of pro-apoptotic proteins such as DP5 and BIM that are both able to induce the intrinsic pathway by either blocking the anti-apoptotic BCL-2 and BCL-XL or by directly activating the pore-forming proteins BAK and BAX. The loss of neurotrophic support is also linked with the loss of PI3K-Akt and MAPK pathways, de-repressing pro-apoptotic players such as BAD or the transcriptional factor FOXO3A.

In addition, the cell death program induced by neurotrophins can be an “active” one, involving the neurotrophin receptors. It is the case of p75<sup>NTR</sup> which following binding of NGF, BDNF or even proneurotrophins is able to induce apoptosis in several neuronal populations. Recent studies imply that in this scenario, p75<sup>NTR</sup> is cleaved releasing an intracellular domain (ICD) that following interaction with TRAF, NRIF or NRAGE becomes highly apoptotic.

This “active” cell death is also the hallmark of the dependence receptors TRKC and TRKA. In the absence of their respective ligands, TRKC is cleaved by caspases releasing a death-inducing fragment, while TRKA seems to require p75<sup>NTR</sup>.

In conclusion, this review is trying to broaden the general perception on neurotrophin limitation-triggered apoptosis that is not just a program “by default” but also an active death signaling initiated by neurotrophin receptors.

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## Review Article

## Neurotrophins and cell death

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## ABSTRACT

The neurotrophins – NGF, BDNF, NT-3 – are secreted proteins that play a major role in neuron survival, differentiation and axon wiring toward target territories. They do so by interacting with their main tyrosine kinase receptors TrkA, TrkB, TrkC and p75<sup>NTR</sup>. Even though there is a general consensus on the view that neurotrophins are survival factors, there are two fundamentally different views on how they achieve this survival activity. One prevailing view is that all neurons and more generally all normal cells are naturally committed to die unless a survival factor blocks this death. This death results from the engagement of a “default” apoptotic cell program. The minority report supports, on the opposite, that neurotrophin withdrawal is associated with an active signal of cell death induced by unbound dependence receptors. We will discuss here how neurotrophins regulate cell death and survival and how this has implications not only during nervous system development but also during cancer progression.

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### Neurotrophins and the neurotrophic factor hypothesis

The neurotrophins are a highly conserved family of secreted proteins that play a major role in neuron survival, differentiation

and axon wiring toward target territories [1,2]. Their incredible adventure started more than 50 years ago when Rita Levi-Montalcini and colleagues discovered the first neurotrophin, the nerve growth factor (NGF), as an essential cue for neuronal survival and axonal growth [3]. It was proposed that neuron

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survival is affected by modifications in target tissue size [4,5] and supported the view that neuron numbers are controlled by the availability of their innervating targets. This set the foundation of the “Neurotrophic Factor hypothesis”. The other neurotrophins, which include the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) were identified years later and completed the picture of an intricate network of survival factors that orchestrate the generation and maintenance of diverse neuronal populations. The neurotrophins exert their effects through binding to two different classes of cell surface receptors, the Trk (A, B and C) tyrosine kinase receptors, and the neurotrophin receptor p75<sup>NTR</sup>, a member of the tumor necrosis factor (TNF) receptor superfamily [2]. While p75<sup>NTR</sup> is able to bind all mature neurotrophins, Trk family members exhibit ligand selectivity: NGF is the preferential ligand for TrkA, BDNF and NT-4 for TrkB and NT-3 for TrkC.

While p75<sup>NTR</sup> is still a puzzling and very debated receptor, it is generally accepted that the main neurotrophin receptors are the Trk receptors and the classic view is that by activating the Trk tyrosine kinase receptors, neurotrophins regulate a large spectrum of functions including the proliferation and survival of neurons, axonal and dendritic growth, cytoskeleton remodeling, synapse formation and maintenance. In addition, neurotrophins also have various roles outside the nervous system, in cardiac development, neovascularization or immune system homeostasis [6]. To mediate this wide variety of functions, the classic accepted scheme is that neurotrophin binding to Trk receptors results in receptor dimerization and autophosphorylation of their cytoplasmic kinase domains; the signaling cascade issued from this ligand/receptor binding have been defined in details and described in numerous elegant reviews. Briefly, Trk phosphorylated residues form critical docking and binding sites for several adaptor proteins that ultimately activate Ras. On its turn, Ras triggers PI3K, the p38 MAPK/MAPK-activating protein kinase 2 and the c-Raf/ERK pathways [6].

The programmed cell death affecting neurons can be classified into *morphogenetic* apoptosis, that occurs during the formation of different neuronal populations, in specific patterns and shapes, and the *pathological* apoptosis that is observed after injury like cerebral ischemia and trauma, or during chronic neurodegenerative diseases, such as amyotrophic lateral sclerosis or Alzheimer's disease. The morphogenetic apoptosis affects between fifty and eighty percents of the neurons that are overly produced or that are unable to correctly innervate their targets [7]. The neurotrophic theory proposed that neurotrophins play a major role in shaping the nervous system by regulating the balance between neuronal survival and apoptosis. Neurotrophin pro-survival effects have been extensively described in many excellent reviews [1,6,8]. However, a point that we would like to discuss here in more details is how this regulation of cell survival and death takes place in the developing nervous system but also through adulthood with possible implication in cancer progression.

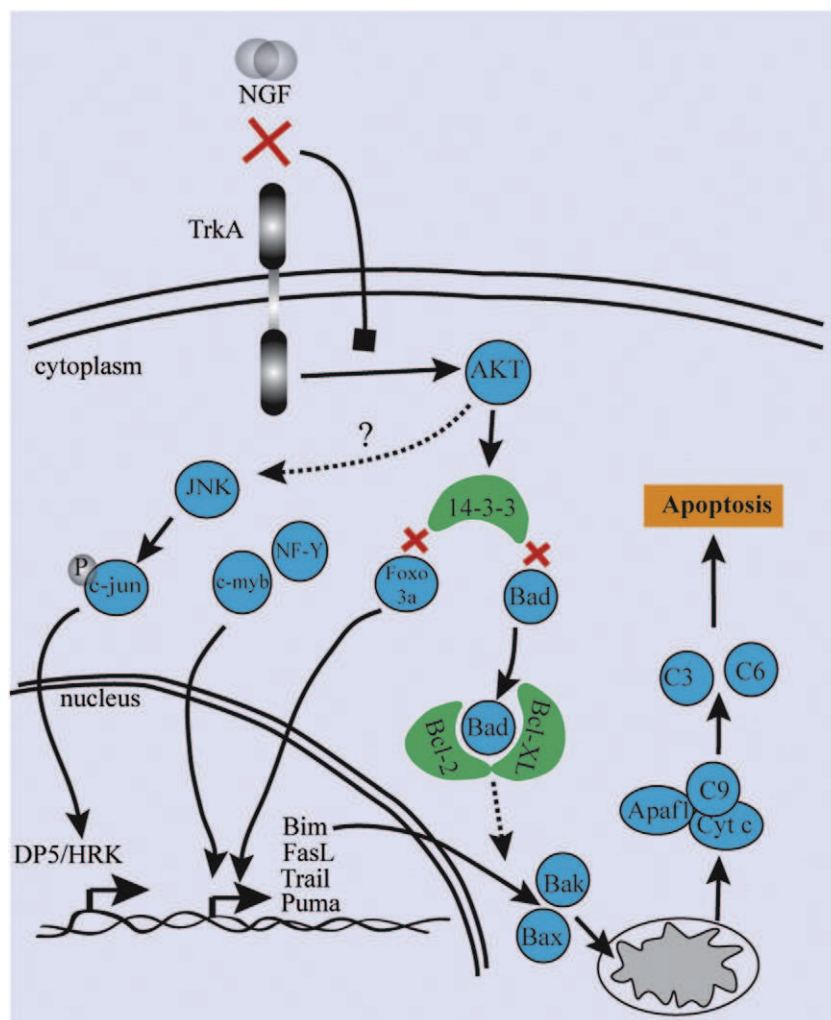
Even though there is a general consensus on the view that neurotrophins are survival factors, two fundamentally different views are currently ruling the neurotrophin field on how they achieve this survival activity. One prevailing view is that based on the Martin Raff hypothesis that all neurons (and more generally all normal cells) are naturally committed to die unless a survival factor blocks this death [9]. This death results from the engagement of a “default” apoptotic cell program (Fig. 1). The minority report supports, on

the opposite, that neurotrophin withdrawal is associated with an active signal of cell death induced by unbound dependence receptors.

## Neurotrophins and inhibition of a default apoptotic program

Since the identification of neurotrophic factors [10], researchers tried to figure out how neurons die in the absence of trophic support. One of the first approaches was done by Martin et al. [11] who used specific inhibitors of both transcription and protein syntheses to show that neuronal death associated with NGF withdrawal requires active RNA and macromolecule assembly. Along the same line, back in early nineties, Garcia et al. showed that the anti-apoptotic protein Bcl-2 is able to rescue sympathetic neurons from NGF withdrawal-induced cell death [12]. Since then, it is classically admitted that cell death observed upon neurotrophin withdrawal occurs through what is now called the intrinsic apoptotic pathway (Fig. 1). Along this line, Bax, one of the targets of Bcl-2, is expressed in the developing nervous system and is required for neuron death since neurons from Bax-deficient mice are protected from apoptosis [13,14]. Moreover, NGF deprivation is associated with Bax redistribution from the cytoplasm to the mitochondria, followed by mitochondria outer membrane permeabilization (MOMP), cytochrome c release and apoptosis [15]. The inhibition of cytochrome c through microinjection of a blocking antibody also rescues the cell death of sympathetic neurons [16]. After the release from the mitochondrial intermembrane space, cytochrome c engages the apoptotic protease activating factor-1 (APAF-1) into a heptameric platform called the apoptosome [17]. The brain hyperplasia, particularly intense in the diencephalon and midbrain, observed in mice invalidated for APAF-1 suggests the important role of this protein in the morphogenetic as well as histogenetic programmed cell death in the developing nervous system. Similar effects on neuron cell death were observed on caspase 3- and 9-null mice suggesting that the intrinsic apoptotic pathway is important in regulating neuronal cell death in the developing brain [18,19]. Along the same line, using cultured sympathetic neurons from wild type and Apaf1<sup>−/−</sup>, caspase-9<sup>−/−</sup> or caspase-3<sup>−/−</sup> mice, it was elegantly shown that Apaf-1, caspase-9 and caspase-3 are essential for NGF withdrawal-associated apoptosis [20].

From these studies, the key question remained however how this intrinsic pathway is activated upon neurotrophin withdrawal. This was actually answered by the extensive works performed on other tyrosine kinase receptors: in the presence of neurotrophins, Trk receptor activation is associated with the activation of the PI3K-Akt and MAPK pathways, two strong survival pathways that block both the intrinsic and extrinsic pathways for apoptosis [21] (Fig. 1). Along this working scheme, when the pro-survival signal mediated by PI3K-Akt is turned-off, BAD is no longer phosphorylated and sequestered by the chaperone protein 14-3-3 and therefore, it is released into the cytoplasm where it then binds to Bcl-2 or Bcl-XL and triggers apoptosis [22,23]. Similarly, the transcription factor forkhead box O3, FOXO3A, is no longer phosphorylated by Akt, is set free from 14-3-3 and is then able to translocate into the nucleus and activate the transcription of proapoptotic genes: FASL [24], Trail, Puma [25] and BIM (BCL-2-interacting mediator of cell death) [26–28]. Bim interacts with the antiapoptotic proteins as well as the effectors, directly inducing BAK and BAX oligomerization and



**Fig. 1 – Death by neurotrophin withdrawal.** The apoptosis induced by neurotrophin withdrawal is best characterized in the case of TrkA. The lack of NGF suppresses the survival signals controlled by PI3K-Akt, finally leading to a de-repression of the pro-apoptotic Bad; on the other hand, the loss of the survival signals is associated with JNK-mediated transcriptional activation of other pro-apoptotic inducers such as DP5, Bim and Puma.

MOMP [17]. More generally, in the absence of Akt activation, CREB and NF- $\kappa$ B survival pathways are silenced [29–31]. BAD and CREB are also targets of the Ras-MAPK-RSK pathway that, synergistically with Akt, activates survival pathways that are repressed in the absence of neurotrophins.

Similarly, many studies have shown that the loss of survival signal in neurons is associated with the activation of the JNK-c-Jun [32–35] even though the link between Trks and this activation is still unclear. However, this pathway appears to link DP5, another important effector of the death program observed after the loss of the survival signal. The DP5 gene was first identified in a genetic screen for genes whose expression is increased during programmed cell death in the NGF-deprived sympathetic neurons [36]. Almost concomitantly, the human ortholog of DP5, called harakiri (HRK) was identified as being able to interact with Bcl-2 and Bcl-XL [37]. The levels of DP5 mRNA were the highest 15 h after NGF removal, this time being consistent with neurons' commitment point to die. Furthermore, the overexpression of DP5 was sufficient to promote cell death even in the presence of NGF and this was partially rescued by Bcl-2. Along with the activation of

the intrinsic pathway, Bax seems to be required for this cell death since DP5 fails to kill Bax deficient neurons [38]. The increase in DP5 mRNA is dependent on the MLK-JNK-c-jun pathway [39]. More specifically, in the absence of NGF, the stress-responsive mixed-lineage kinase (MLK) and JNK protein kinase are activated leading to c-jun phosphorylation and activation; the activated c-jun will further bind and increase the activity of DP5 promoter immediately after neurotrophic factor withdrawal. The expression of Bim, a Bcl-2 pro-apoptotic family member, also seems to be directly induced by the JNK pathway [28]. BIM is also activated by the CDK4-E2F-MYB axis following NGF withdrawal, this requiring C-MYB binding to BIM promoter [40].

### **p75<sup>NTR</sup> paradigm: a trophic killer or a dependence receptor**

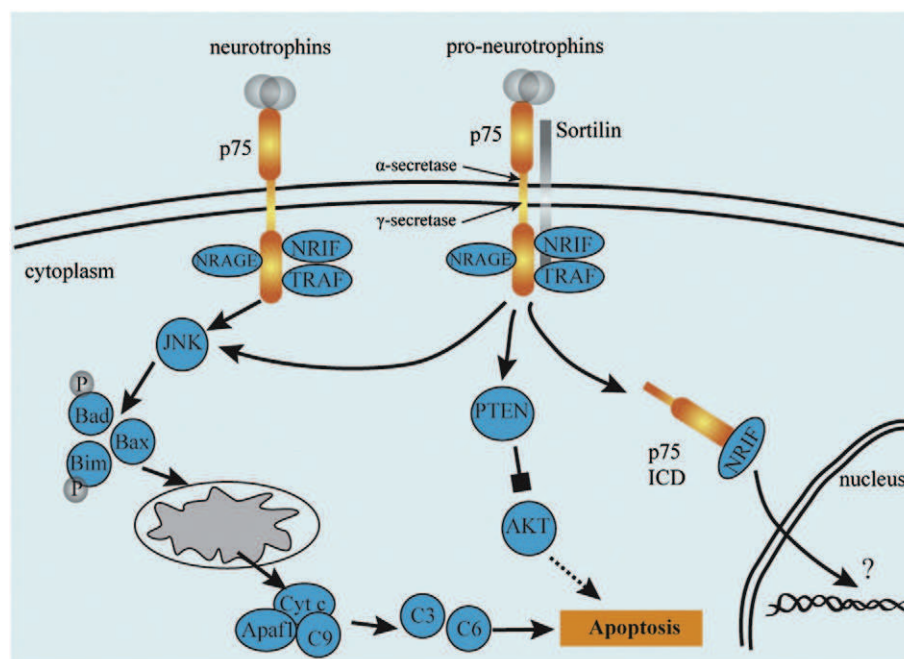
In the late eighties, searches for a receptor for NGF, led to the discovery of two distinct receptors, p75<sup>NTR</sup> [41,42] and TrkA [43]. TrkA was shown to be capable of mediating the known responses



to NGF, such as neurite outgrowth and neuronal survival (for reviews [44,45]). These studies initially left the role of  $p75^{\text{NTR}}$  unexplained. Subsequent studies demonstrated that  $p75^{\text{NTR}}$  and TrkA collaborate to produce high-affinity sites for NGF binding [43], and that  $p75^{\text{NTR}}$  expression may enhance the selectivity of neurotrophin binding for specific Trks (TrkA, B, and C; [46]). However,  $p75^{\text{NTR}}$  was also shown to have Trk-independent effects.  $p75^{\text{NTR}}$  is a member of a superfamily of the so-called death receptors that include the tumor necrosis factor receptors and Fas [47] (Fig. 2). The consideration that  $p75^{\text{NTR}}$  ligand is a survival factor and that receptors close to  $p75^{\text{NTR}}$  were pro-apoptotic proteins led Dale E Bredesen and S. Rabizadeh to hypothesize that  $p75^{\text{NTR}}$  could be a sort of anti-death receptor that would kill neurons upon NGF withdrawal rather than upon ligand binding. They showed using primary neuron culture and transient transfection that the expression of  $p75^{\text{NTR}}$  induced apoptosis when  $p75^{\text{NTR}}$  was unoccupied by NGF, whereas binding of NGF blocked apoptosis [48–50]. The finding that  $p75^{\text{NTR}}$  expression induces apoptosis in the absence of ligand, but inhibits apoptosis following ligand binding, suggested that  $p75^{\text{NTR}}$  expression creates a state of cellular dependence on NGF (or other  $p75^{\text{NTR}}$  ligands). These data supported the view that an unbound receptor could trigger apoptosis and was the premise of the dependence receptor paradigm. This paradigm proposes that a functional family of receptors shares the ability to trigger apoptosis in the absence of their respective ligand. So far 20 receptors, even though they do not necessarily show structural similarities, are dependence receptors and as such create a state of cellular dependence on their respective ligand [51]. This pro-apoptotic activity upon ligand withdrawal has been shown for receptors such as DCC and patched to be important to both tune the developing nervous system [52,53] and to negatively regulate tumor progression [54–56].

Yet, even though the seminal 1993 work on  $p75^{\text{NTR}}$  as a pro-apoptotic receptor when unbound by its ligand [48] was the stepping-stone of what is now called “the dependence receptor paradigm”, it is fair to say that these data raised skepticism among the neurotrophin field and several following works rather supported the view that  $p75^{\text{NTR}}$  behaves as a more classic death receptor triggering cell death in the presence of its ligands NGF or BDNF (Fig. 2). Indeed, NGF or BDNF activation of  $p75^{\text{NTR}}$  has been reported to induce neuronal cell death. It was thus proposed that  $p75^{\text{NTR}}$  mediates together with TrkA the survival of neurons during the establishment of target innervations while it induces apoptosis in the early postnatal period [49]. NGF was shown to act as a pro-apoptotic ligand of  $p75^{\text{NTR}}$  for a large variety of cells including the neurons in the isthmo-optic nucleus, the retinal ganglia cells or the mature oligodendrocytes [57–60]. It was thus demonstrated that all four neurotrophins induce death of hippocampal neurons through  $p75^{\text{NTR}}$  binding [61]. The neurons that were particularly sensitive to this cell death were the ones lacking the Trk receptors, confirming that Trk expression protects neurons from neurotrophin-induced cell death [62]. Friedman and colleagues also described JNK pathway as being responsible for  $p75^{\text{NTR}}$ -induced cell death. Several reports have shown that  $p75^{\text{NTR}}$ -dependent JNK activation leads to the phosphorylation and activation of Bad and Bim, both being able to induce the mitochondrial translocation of Bax and further the release of cytochrome c and activation of caspase-3, -6 and -9 (reviewed in [63]).

An unexpected layer of complexity was added when Hempstead and colleagues revealed that the unprocessed forms of the neurotrophins (proneurotrophins) are high affinity ligands for  $p75^{\text{NTR}}$ . They also found that binding of proneurotrophins to  $p75^{\text{NTR}}$  induces cell death [45]; this was later confirmed in numerous cell types including corticospinal neurons, oligodendrocytes,



**Fig. 2 – Death by neurotrophins – the case of p75.** Both mature and pro-neurotrophins were described to bind  $p75$  and induce a JNK-dependent intrinsic apoptosis, similar to TrkA. This is modulated by the co-receptor sortilin and also specific intracellular interacting proteins such as NRAGE, NRIF and TRAF; the  $\gamma$ -secretase cleavage seems to be crucial for the  $p75$ -induced cell death.

Schwann cells or sympathetic neurons [64]. Interestingly, in order to induce neuronal cell death, the proneurotrophins need to bind sortilin, an adaptor receptor that specifically recognizes the pro-domains of proNGF and proBDNF. In this way, sortilin serves as a switch that enables p75<sup>NTR</sup>-expressing neurons to respond to neurotrophins and to undergo apoptosis rather than survival [65] (Fig. 2).

The p75<sup>NTR</sup>-induced apoptosis in the presence of proneurotrophins seems to require multiple mechanisms. The *in vivo* investigation of the role of proneurotrophins in neuronal apoptosis revealed that proneurotrophins are produced in the brain in pathological conditions and induce the cell death of basal forebrain (BF) neurons; this coincides with JNK phosphorylation and cleavage of caspase-6 and -3, Trk activation being unable to protect in this context the BF neurons from pro-NGF-induced cell death [66]. Furthermore, pro-NGF is able to induce PTEN, thus preventing the activation of Akt by BDNF and leading to cell death [67].

Recent studies found that ligand-induced p75<sup>NTR</sup>-mediated apoptosis requires a ligand dependent cleavage of the receptor by  $\gamma$ -secretase which releases the intracellular domain (ICD). This processing results in the nuclear translocation of NRIF, a zinc finger DNA binding protein [68]. This nuclear shuttling is dependent on TRAF, another interacting partner of p75<sup>NTR</sup> ICD [69]. How NRIF nuclear translocation potentiates cell death is still unknown, but there are speculations about a putative transcriptional activation of pro-apoptotic genes [70]. Furthermore, NRAGE, a member of the MAGE (melanoma antigen) family, also interacts with p75<sup>NTR</sup> to mediate neurotrophin-induced cell death through a mechanism involving mitochondrial cytochrome c release and activation of caspases-9, -3 and -7. Moreover, NRAGE-dependent apoptosis requires JNK and subsequent phosphorylation and accumulation of c-Jun [71].

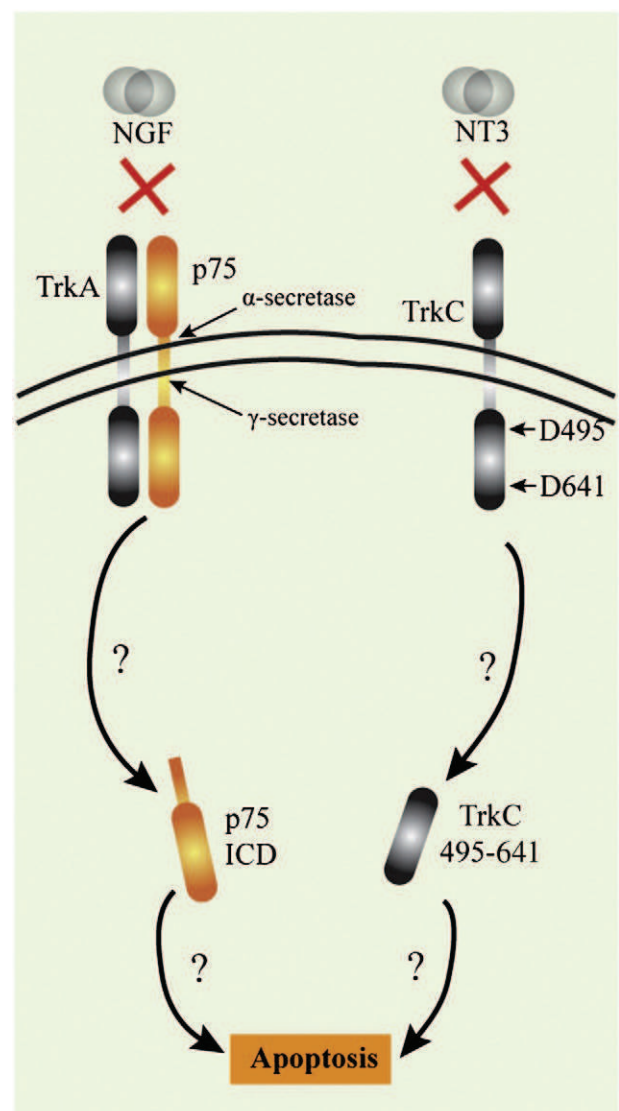
From the initial works demonstrating p75<sup>NTR</sup> as a pro-apoptotic receptor in the absence of neurotrophin binding to more recent works on p75<sup>NTR</sup> as a more classic death receptor when engaged by pro-neurotrophins, all these studies support the view that p75<sup>NTR</sup> is a strong pro-apoptotic trigger which integrates the type of neurotrophin present in the milieu, the cell population and the type of receptors and adaptor proteins expressed to determine the ultimate fate of the cell. It is yet fair to say that up to a few years ago, the general feeling in the neurotrophin community was that dependence receptors did not mean much in the massive cell death observed upon neurotrophin limitation.

### TrkC and TrkA: when dependence receptors strike back

However, as very often in science, the minority report may actually turn as not so unattractive after all. Indeed, while trying to extend the dependence receptor family, our group screened for the ability of intracellular domains of transmembrane receptors to be cleaved *in vitro* by caspase, a trait observed in most dependence receptors so far. We thus picked TrkC as a putative caspase cleaved receptor and we investigated whether it could behave as a dependence receptor. We showed that TrkC could actively trigger apoptosis in the absence of its ligand NT-3 in various cell lines (Fig. 3). The mechanism behind this active death signaling appears to start with a cleavage by a first line of locally activated caspases at two sites leading to the release of a pro-apoptotic domain. This death appears to be dependent on caspase-9 but not on caspase-

8 [72]. More importantly, in collaboration with Urmas Arumae, we showed that primary neurons cultured in the absence of NT-3 do not undergo apoptosis – as expected with a death by default – unless unbound TrkC is allowed to trigger apoptosis [72].

Interestingly, Yves-Alain Barde and his team confirmed that TrkC induces cell death in the absence of NT-3, using an elegant model of engineered embryonic stem (ES) cells *in vitro* and *in vivo*. Surprisingly, in their experimental model, TrkA was also able to induce apoptosis in the absence of NGF (Fig. 3). More specifically, *TrkA*, *TrkB* and *TrkC* cDNAs were expressed under the control of the neuron-specific microtubule-associated protein tau (*Mapt*) locus in ES cells that were driven to neuronal differentiation into glutamatergic neurons. These neurons have almost physiological levels of the Trk proteins. They showed that ES-cell-derived neurons that express TrkA and TrkC, die *in vitro* in the



**Fig. 3 – TrkC and TrkA as dependence receptors.** Newly described as dependence receptors, TrkA and TrkC trigger apoptosis when their respective ligand is withdrawn. To do so, TrkC is releasing a pro-apoptotic fragment from a double-caspase cleavage while TrkA requires the recruitment of the pro-apoptotic p75<sup>NTR</sup>.



absence of ligand, and that their death is reversed in the presence of NGF and NT-3 respectively, whereas TrkB-expressing neurons survive in the absence of BDNF. A tetraploid complementation system was then used to generate embryos entirely derived from the engineered ES cells. In these embryos, GFP was also expressed under the control of the *tau* promoter, allowing visualization of the whole nervous system. At E13.5, the nervous system of TrkA and TrkC-expressing embryos suddenly disappears, whereas the TrkB-expressing embryos maintain their nervous system intact [73]. Thus, from Barde's work and our work, it appears that classic survival receptors are, in fact, dependence receptors. It opens then the view that the death observed upon neurotrophin limitation is not a "by default" program but rather an active death signaling triggered by the neurotrophin receptors.

Numerous points however are raised by these two studies: the first one is that we initially missed in our screen of putative dependence receptors, TrkA. Indeed, TrkA, unlike TrkC, is not cleaved by caspase. However Barde demonstrated that TrkA does not need a cleavage by caspase to be pro-apoptotic, thus suggesting that different downstream mechanisms could be used by dependence receptors to trigger apoptosis. Along this line, it has been shown that while the dependence receptor Patched triggers apoptosis by recruiting a caspase-activating complex named "dependosome" and including caspase-9 [74], the dependence receptor UNC5H2 induces apoptosis through activation of DAPK, a serine threonine kinase [75,76]. Thus even though in this two latter cases both receptors show a caspase cleavage site in their intracellular domain, it may not be a pre-requisite for death induction by a dependence receptor. In the case of TrkA, Barde and colleagues showed some interesting observations suggesting that death induced by TrkA requires the recruitment of p75<sup>NTR</sup> to TrkA and is mediated through p75<sup>NTR</sup> (Fig. 3). This observation actually recalls the data shown by Bredesen and colleagues who observed that forced expression of p75<sup>NTR</sup> triggers apoptosis in the absence of ligand [48]. From this, two hypotheses could be proposed: either both TrkA and p75<sup>NTR</sup> are dependence receptors and actively support cell death when expressed together at the surface of the cell or, as favored by Barde and colleagues, p75<sup>NTR</sup> is not a dependence receptor but rather the "hired" killer of the dependence receptor TrkA. At this stage, it would also be interesting to test whether p75<sup>NTR</sup> could also trigger the death induced by TrkC, supporting the view that TrkC induces cell death in the absence of NT-3 both via a direct release of a pro-apoptotic domain and via the recruitment of p75<sup>NTR</sup>.

At first glance, researchers outside the neurotrophin field could say that "death by default" or death by dependence receptor is mainly a semantic issue, as at the end, the cell undergoes apoptosis in the absence of its ligand. However, this, we believe, has considerable importance when diseases are involved. Indeed, a large effort of the last 20 years has been made to develop drugs which inhibit kinases – e.g. Imatinib/Gleevec – with the overall certitude that, by blocking kinase survival pathways, tumor cells should undergo apoptosis. However, the dependence receptor paradigm related to neurotrophin receptors and also to more recently identified tyrosine kinase dependence receptors [77,78] supports the view that "therapeutic" cell death will be achieved only when unbound TrkC, TrkA and/or p75<sup>NTR</sup> will actively trigger apoptosis. It is thus interesting to note that expressions of TrkA and TrkC are good prognosis-associated markers in neuroblastoma, a pediatric

cancer originating from the neural crest lineage, whereas TrkB is associated with poor prognosis (for review [79]). Thus expression of Trk dependence receptors appears as a constraint for tumor progression while expression of a Trk "trophic-only" receptor is rather pro-oncogenic. Thus dependence receptor-induced apoptosis may potentially be used to trigger tumor cell death and possibly tumor regression. Along this line we have indeed shown that the ability of TrkC to trigger apoptosis is bypassed in a large fraction of aggressive neuroblastoma by acquiring an autocrine production of TrkC ligand, NT-3. By interfering with NT-3/TrkC interaction, we were able to trigger TrkC-dependent tumor cell death *in vitro* and tumor growth and metastasis inhibition in chicken and mice models [80]. This supports the view that efficient treatment of cancer may require a combination of treatments based on a kinase inhibitor to shut-down survival pathways and a drug inhibiting Trk/ligand interaction to trigger dependence receptor-mediated apoptosis. Thus, neurotrophins and their survival activity which was so impressively put forward by several generations of neurobiologists as a key determinant for nervous system development may turn as a central mechanism regulating cancer progression.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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#### **4.2. Annex 2: The histone acetyltransferase component TRRAP is targeted for destruction during cell cycle (Research article, accepted in Oncogene)**

In this article that took shape during my Master internship in the Epigenetics Team (International Agency for Research on Cancer, Lyon) and was accepted for publication at the end of my thesis, we describe that TRRAP, a key component of histone acetyltransferase (HAT) complexes, is degraded in cell cycle-dependent manner. We show that TRRAP degradation is mediated by the APC/C ubiquitin ligase activators Cdc20 and Cdh1. TRRAP is clearly degraded at the beginning of mitosis and the ectopic expression of both Cdc20 and Cdh1 reduce drastically TRRAP protein levels. TRRAP overexpression that would mimic the inability of cells to degrade this epigenetic regulator results in mitotic failure, characterized by lagging chromosomes, chromosome bridges and multipolar spindles. These defects are related with an increase of global histone H4 hyperacetylation, a histone mark associated with the ability of chromosomes to condense while entering mitosis.

These results strongly suggest that TRRAP is targeted for degradation in mitosis in order to allow proper chromosome condensation and segregation into daughter cells.

# **The histone acetyl transferase component TRRAP is targeted for destruction during the cell cycle**

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**Running title:** TRRAP proteolysis during the cell cycle

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## **Abstract**

Chromosomes are dynamic structures that must be reversibly condensed and unfolded to accommodate mitotic division and chromosome segregation. Histone modifications are involved in the striking chromatin reconfiguration taking place during mitosis. However, the mechanisms that regulate activity and function of histone modifying factors as cells enter and exit mitosis are poorly understood. Here, we show that the anaphase-promoting complex or cyclosome (APC/C) is involved in the mitotic turnover of TRRAP, a common component of histone acetyltransferase (HAT) complexes and that the pre-mitotic degradation of TRRAP is mediated by the APC/C ubiquitin ligase activators Cdc20 and Cdh1. Ectopic expression of both, Cdh1 and Cdc20 reduced the levels of co-expressed TRRAP protein and induced its ubiquitination. TRRAP overexpression or stabilization induces multiple mitotic defects, including lagging chromosomes, chromosome bridges, and multipolar spindles. In addition, lack of sister chromatid cohesion and impaired chromosome condensation were found after TRRAP overexpression or stabilization. By using a truncated form of TRRAP, we show that mitotic delay is associated with a global histone H4 hyperacetylation induced by TRRAP overexpression. These results demonstrate that the chromatin modifier TRRAP is targeted for destruction in a cell cycle-dependent fashion. They also suggest that degradation of TRRAP by the APC/C is necessary for a proper condensation of chromatin and proper chromosome segregation. Chromatin compaction mediated by histone modifiers may represent a fundamental arm for APC/C orchestration of the mitotic machinery.

**Keywords:** APC/ Cdc20/ Cdh1/ TRRAP/ mitosis/ cell cycle



## Introduction

Accurate transmission of genetic information requires chromatin reconfiguration from a relaxed to a highly condensed structure that facilitates cell division. Genomic DNA is wound around histones, that are subject to various post-translational modifications that dictate its level of compaction (1). Specifically, histone acetylation, is involved in chromatin reconfiguration as cells enter and exit mitosis (2, 3). Mitosis is characterized by hypoacetylation of histones, with the acetylation of histones decreasing during the early stages of mitosis, absent in metaphase and anaphase, and then gradually increasing again in the late mitotic phase (3). The anaphase-promoting complex/cyclosome (APC/C) is a multicomponent E3 ubiquitin ligase that, by targeting protein substrates for 26S proteasome-mediated degradation through ubiquitination, coordinates the temporal progression of eukaryotic cells through mitosis (4). Although the role of the APC/C in targeting mitotic players is clearly established, the mechanism that couples the activity of histone acetylation complexes with cell cycle transitions is poorly understood.

Acetylation of core histone tails by histone acetyltransferases (HATs) is a paradigm for transcriptional activation and is reversed by histone deacetylases (HDACs) (5, 6). A common component of many HATs is the TRansformation/tRanscription domain-Associated Protein (TRRAP), whose function has been linked with the recruitment of HAT complexes, both from GNAT and the MYST HAT families, to transcription factors bound to their target promoters in chromatin (reviewed in (7)). By regulating histone acetylation, TRRAP is believed to participate in all processes related with the level of compaction of chromatin, including the induction of transcription (8), and the repair of DNA double strand breaks (9, 10). It was shown that TRRAP deficiency had consequences in cell division. Homozygous

mutation of Trrap in mouse germline results in peri-implantation lethality, whereas embryonic stem cells derived from Trrap knock-out mice show different types of chromosomal aberrations and mitotic defects (11). Moreover, TRRAP was shown to be involved in transcriptional regulation of genes involved in mitotic checkpoint, and mitotic progression (12, 13). Finally, TRRAP protein was also shown to participate in the activation of histone transcription in early S phase (14). Dynamic changes in chromatin reconfiguration during cell cycle progression require concerted efforts of histone modifying machineries. However, the mechanism that couples the activity of HAT complexes with cell cycle transitions is poorly understood.

Here, we studied the regulation of TRRAP during the cell cycle and revealed the mechanism underlying cell cycle TRRAP proteolysis, and the consequences of failure to destroy the protein during entry in and exit from mitosis.

## **Results**

### ***TRRAP protein expression is regulated during the cell cycle***

Previous work from our group has implicated TRRAP protein in the control of cell cycle and mitotic progression (12). Because many proteins involved in cell cycle and mitotic control are themselves expressed in a periodical fashion, we were interested in studying the regulation of TRRAP during the cell cycle. To this end, we arrested 293T cells at the G1/S boundary using a double thymidine block followed by a release into fresh media to allow cells to progress through the cell cycle. Whole cell extracts were prepared to monitor expression of TRRAP by western blotting. After thymidine block TRRAP expression is higher compared to asynchronous cells (Figure 1A). Protein levels remain high up to 5 hours after release,



corresponding to S phase. Before mitosis (approximately 8 hours post-release), TRRAP protein expression drops sharply and remains at low levels until the following late G1 phase. Consistent results were obtained in an independent cell line, HeLa (Figure 1B). In this case, peak TRRAP levels were reached during S phase (4 to 7 hours post-release from thymidine block) (Figure 1B). In addition, an alternative protocol of synchronization was used to further explore the kinetics of TRRAP turnover. To this end, a prometaphase arrest was induced by treating HeLa cells with nocodazol. Cells arrested in this way displayed lowest levels of TRRAP for the first hours after release (Supplementary Figure 1). This was followed by a marked increase at 8-10 hours postrelease, approximately the time when cells reached the G1/S phase transition.

To precisely define cyclical expression of TRRAP, bromodeoxyuridine (BrdU) incorporation was used to quantify the percentage of cells in S phase. In addition, phosphorylation of histone H3 in serine 10 (pS10H3), was used for mitotic index (MI) quantification after cell synchronization. As shown in Figure 1B, protein levels began to drop after BrdU incorporation ceased, and concomitantly with an increase in pS10H3 signal. Moreover, TRRAP protein levels begin to drop at approximately one hour before CCNB1 degradation in both, 293T and HeLa cells (Figure 1A). Of notice, TRRAP mRNA expression was not significantly regulated during the cell cycle, as assessed by qRT-PCR (data not shown). An additional double thymidine synchronization with hourly time points and densitometry quantification was done in HeLa cells (Figure 1C). This synchronization confirmed that TRRAP levels are maximal during S phase, and begin to drop before mitosis. As can be seen, TRRAP initial reduction starts before both, Cyclin A and B1. Low levels of TRRAP are still detectable after mitosis. In contrast, the HAT component Tip60 (a common TRRAP partner)

continues to be expressed during the whole cell cycle. Together, these results indicate that TRRAP protein is expressed in a cell cycle dependent fashion, with highest expression levels during S phase and a drop prior to mitotic exit. The lack of significant changes at the mRNA level suggests a post-transcriptional control of TRRAP expression.

***TRRAP protein is degraded by ubiquitination prior to mitotic exit***

Proteasome-dependent degradation is a common mechanism to control protein levels during the cell cycle. To test whether TRRAP downregulation was dependent on the Ubiquitin-Proteasome System (UPS), we investigated the ubiquitination status of TRRAP using immunoprecipitation. Constructs of Flag-TRRAP and empty-vector were transfected into either HeLa or 293T cells and 48 hours post-transfection lysates were prepared and immunoprecipitated using anti-Flag antibody. Western blot analysis using anti-ubiquitin antibody revealed higher molecular weight TRRAP bands in both HeLa and 293T cells at similar levels, consistent with TRRAP ubiquitination (Figure 2A). We further tested the effects of proteasome function on the accumulation of TRRAP protein. As observed in the initial experiments, prometaphase cells display reduced levels of TRRAP protein in comparison to asynchronous cells (Figure 2B). This striking reduction of TRRAP protein levels in nocodazol-treated cells was prevented by the proteasome inhibitor MG132 (Figure 2B). These results suggest that the drop in TRRAP protein levels is due to increased proteolysis in mitotic cells.

To further validate these results, HeLa cells were transfected with a GFP-tagged TRRAP-expressing vector (GFP-TRRAP) and TRRAP protein levels were evaluated by fluorescent microscopy in the presence of proteasome inhibitor. As shown in Figure 2C, expression of GFP-TRRAP was increased after as short as 4 hours of treatment with MG132 (mean

fluorescence per nucleus = 19.2 and 30.4 in control and MG132-treated cells, respectively.  $P$  value = 0.037). To follow the accumulation of TRRAP at different time points after proteasome inhibition, the GFP-TRRAP construct was used in a G1/S synchronization experiment with double thymidine block. Cells were released from the thymidine block and followed for different time points in presence or absence of MG132-induced proteasome inhibition. Accumulation of TRRAP protein occurred at 4 hours after release (i.e. during S phase), although this was much more evident in the MG132 treated cells. As expected from our previous double-thymidine experiments (compare with Figure 1A and 1B), 8 hours after release there was a drop in protein expression (Figure 2D). However, cells treated with MG132 exhibited GFP-TRRAP stabilization up to 8 hours after release (Figure 2D). Together, these data shows that TRRAP protein is ubiquitinated and degraded before mitotic exit by a mechanism that is dependent on the UPS.

### ***Cdc20 silencing induces TRRAP protein accumulation***

Several ubiquitin ligases are involved in the timely destruction of cell cycle proteins at specific cell cycle transitions. To test whether mitosis-specific degradation of TRRAP is mediated by the anaphase promoting complex/Cyclosome (APC/C), the major ubiquitin-ligase regulating the M/G1 transition (15), we analysed TRRAP amino acid sequence and found a destruction motif (D-box) at residue 1583 (RXXLXXXXN) immediately followed by a putative KEN-box motif at residue 1664 (KENXXXXN) (Figure 3A). Target specificity for APC-mediated ubiquitination is dictated by one of two possible APC activators, Cdc20 or Cdh1, and the presence of destruction motifs in its target proteins (4). In contrast to the KEN-box motif, the D-box motif of TRRAP is well conserved in other species analysed, suggesting a biological meaning for its presence (Figure 3A). Because TRRAP harbours

conserved D-box motif and TRRAP degradation occurs prior to mitotic exit, we next examined the stability of TRRAP after specific knock-down of Cdc20 with siRNA interference. Efficient knock-down of Cdc20 in 293T cells by siRNA was verified by qRT-PCR and western blot (Figure 3B and data not shown). As shown in Figure 3B, TRRAP protein was drastically accumulated after Cdc20 siRNA transfection, as compared to a non-targeting siRNA transfection. Moreover, we observed an accumulation of TRRAP protein in Cdc20 depleted cells synchronized in G2/M phase (Figure 3B). Co-immunoprecipitation experiments showed a potential interaction of TRRAP with Cdc20. Both, endogenous TRRAP or transfected Flag-TRRAP, were able to immunoprecipitate Cdc20 in asynchronous HeLa and 293T cells (Figure 3C). This was confirmed with anti-TRRAP and anti-Flag antibodies (Figure 3C, left panel), while no interaction between TRRAP and Cdc6 was observed. Furthermore, when using synchronized cell populations, we found that TRRAP-Cdc20 interaction is cell cycle dependent, with highest interaction during the G2/M transition (after 9 hours of 2T-block release), consistent with the time of TRRAP degradation (Figure 3C, right panel). No interaction was detected between TRRAP and Cdc27, a core component of the APC/C complex, suggesting that APC may target TRRAP specifically via Cdc20. These data suggest a direct and cell cycle stage specific interaction between TRRAP and Cdc20, and favours the idea that TRRAP degradation before mitosis is dependent on APC/C-Cdc20.

#### ***APC/C ligases Cdc20 and Cdh1 target TRRAP protein for degradation***

To validate the activity of the two most important APC/C ligases in TRRAP proteolysis, full length TRRAP was co-transfected Cdh1 or Cdc20 ligases into 293T cells, and TRRAP protein degradation was monitored by Western blotting. Different concentrations of the

ligases were used to define any potential dose/response effect. As shown in Figure 4A (left panel), Cyclin B1, a known target of the APC/C, displayed the expected lower expression after co-transfection with Cdh1. No effect was observed with Cdc20, probably because of the lower stability of the Cdc20 construct. However, either Cdh1 or Cdc20 co-transfections resulted in a dramatic reduction of TRRAP protein levels, with Cdh1 exhibiting a stronger effect (Figure 4A, right panel). Next, we co-transfected 293T cells with plasmids expressing TRRAP and Cdc20 or Cdh1 for 24 hours, and then treated cells with cycloheximide for 1 to 5 hours to inhibit protein synthesis. Cyclin B1 was stable during the 5 h of treatment, while this was not the case in the presence of any of the two ligases (Figure 4B). In a similar way, TRRAP was relatively stable when expressed alone, indicating that TRRAP's half-life is greater than 5 h (Figure 4B). However, in the presence of both, Cdh1 and Cdc20, the half-life of TRRAP was markedly reduced. Densitometry analysis confirmed that both, Cyclin B1 and TRRAP are degraded in the few hours following cycloheximide treatment. Specifically, TRRAP levels were reduced to approximately 30% of basal levels after 4 hours of cycloheximide exposure (Figure 4C). In addition, these data indicate that TRRAP turnover takes place at a slower rate than Cyclin B1.

Together, these data suggest that the APC/C complex plays a role in ubiquitin-mediated turnover of TRRAP. By inducing TRRAP proteolysis, the APC/C ligases Cdc20 and Cdh1 have an important role during this process.

#### ***The Dbox destruction motif in TRRAP is not required for TRRAP proteolysis***

To validate the interaction between TRRAP and the Cdc20 and Cdh1 ligases, we performed co-transfection experiments in 293T cells. Full length TRRAP (GFP-TRRAP) was co-transfected with each of the ligases for 48 hours, followed by immunoprecipitation with anti-

GFP antibody (Figure 5A). This experiment confirms that TRRAP is able to interact with both of the ligases. In addition, it suggests that ubiquitination of TRRAP is higher after co-transfection with Cdh1 and Cdc20 in comparison to TRRAP alone (Figure 5A), consistent with the notion that these ligases are involved in proteolysis of TRRAP.

We next studied the importance of the destruction motifs in TRRAP degradation using site-directed mutagenesis. To this end, we replaced the 3 conserved residues in TRRAP D-box motif (Dbox mutant) or deleted both, D-box and KEN-box motifs (DK mutant) (Figure 3A). Although D-box mutation partially compromised the TRRAP-Cdc20 interaction (Supplementary Figure 2A) and TRRAP oscillation during the cell cycle (Supplementary Figure 2B), TRRAP wild-type and mutant were similarly accumulated after proteasome inhibition (Supplementary Figure 2C). Moreover, Dbox and DK TRRAP mutants were sensitive to Cdh1- and Cdc20-induced proteolysis after ectopic expression (Supplementary Figure 5B), suggesting that the D-boxes have only a partial function in regulating TRRAP destruction by the APC/C.

Together, these results suggest that although Cdc20 and Cdh1 can directly interact with TRRAP, the Dbox/KEN motifs in TRRAP may not play a central role in Cdc20/Cdh1 targeting and destruction of TRRAP. Instead, the presence of other less conserved destruction motifs may be important for TRRAP destruction before mitosis.

***TRRAP overexpression delays mitotic progression and impairs chromosome condensation and sister chromatids cohesion***

A limited number of proteins have been described as targets of the APC/C complex, all of which have been associated with different roles during the cell cycle, most notably in mitotic

exit (15). Therefore, we aimed to test the importance of TRRAP degradation before mitosis by saturating the cellular degradation machinery through TRRAP overexpression. To this end, HeLa and 293T cells were synchronized and mock transfected or transfected with GFP-TRRAP, and cell cycle progression was monitored by time-lapse microscopy (Supplementary Figure 3A). In addition to fluorescence detection, TRRAP overexpression was verified by qRT-PCR and western blot (data not shown). While mock-transfected cells finished cell division in less than 15 minutes after the formation of the metaphase plate, TRRAP overexpressing-cells took up to one hour or longer to complete mitosis, and sometimes cytokinesis was not seen even after 3 hours from chromosomal condensation (Supplementary Figure 3B and 3C). Quantification of this data is presented in Supplementary Figure 3D). Importantly, this delay was frequently accompanied by segregation defects, including chromosome bridges and aberrant cytokinesis (Supplementary Figure 3C).

As the delay in mitotic progression and mitotic exit is often caused by chromosomal segregation errors, we next examined the state of chromosomes in mitotic cells overexpressing TRRAP. To this end, cells were mock transfected or transfected with wild-type TRRAP or D-box mutant, and chromosome segregation was monitored by fluorescence microscopy. Our results revealed that chromosome bridges, lagging chromosomes, and multipolar spindles were a common finding after TRRAP overexpression, compared to mock transfected cells (Figure 6A and 6B), whereas the distribution of cells in the different phases of mitosis was similar in all conditions (data not shown). These segregation defects were observed both, after nocodazol or double-thymidine synchronizations, ruling out a defect associated with drug treatment (Figures 6B and 6C). Similar results were obtained after transfection with the Dbox mutant. In addition, the mitotic index in the presence of nocodazol



was similar between TRRAP-overexpressing cells and control cells (data not shown), ruling out the possibility that the segregation defects observed in TRRAP-overexpressing cells are due to cellular inability to induce a proper spindle-assembly checkpoint (SAC). Therefore, TRRAP overexpression or stabilization results in aberrant mitotic exit and impaired chromosome segregation, in the presence of an intact SAC.

Because acetylation of core histones is considered as a mechanism that dictates the level of relaxation/condensation of chromatin, we reasoned that deficiencies in compaction of chromosomes in cells overexpressing TRRAP may result in segregation defects. To test this possibility, we examined the level of chromatin condensation and chromatin cohesion on metaphase spreads from cells overexpressing TRRAP. Spreads were classified as normal, with increased length of metaphase chromosomes, or different degrees of lack of chromatid cohesion (i.e. partially separated or scattered) (Figure 6D). While no defects in the number of chromosomes were observed in the spreads (data not shown), there were marked differences in the length and cohesion of sister chromatids after transfection with wild-type and D-box mutant TRRAP (Figure 6D and data not shown). Both, a lack of cohesion and increased length of chromosomes were significantly overrepresented in TRRAP transfected cells in comparison to mock transfected controls ( $P<0.05$ ) (Figure 6D). This suggests that the defects in segregation after TRRAP stabilization, including delayed mitosis and chromatin bridges, may be at least partially explained by a defect in condensation and reduced chromatid cohesion. Together, these results show that TRRAP overexpression results in a consistent delay in mitotic progression that could be explained by segregation defects.

### ***TRRAP-related mitotic delay is partially dependent on global histone hyperacetylation***

Functionally, TRRAP is involved in the recruitment of multiple histone acetyl-transferase components. Therefore, we next examined the role of histone modifications in the mitotic defects observed in TRRAP overexpressing cells. We found an increased H4 acetylation in the DNA-bound fraction of chromatin fractionated DNA after transfection with full-length TRRAP (Figure 7A). Interestingly, we also found a marked increase in H3K9me3 and a moderate increase in H3K4me2, suggesting that TRRAP overexpression induces changes in other histone modifications in addition to acetylation. No changes were found for other histone marks (histone H3, H3K12Ac or H3K9Ac) or the heterochromatin protein HP1 (Figure 7A).

TRRAP-mediated histone acetylation may have a direct link to the mitotic defects described above. To directly address this possibility we designed a truncated version of TRRAP (tTRRAP) that has a limited ability to induce histone acetylation. To this end, we introduced two stop codons downstream of the putative destruction boxes (Dbox and KEN box) (Figure 7B). As expected, this C-terminal-truncated form of TRRAP is still subjected to ubiquitination (Figure 7C). In addition, we show that, at similar levels, this truncated form of TRRAP is indeed unable to increase global histone H4 acetylation, confirming an efficient elimination of the critical domain involved in HAT activity (Figure 7D). To explore the impact of the truncated form of TRRAP on mitosis, we co-transfected H2b-cherry and either full length TRRAP or tTRRAP in HeLa cells during 48 hours. Nocodazol (100 ng/ml) was added for the last 16 hours, and time lapse microscopy was performed after release. The number of mitosis (per 100 cells) was determined during the next 30 minutes following nocodazol release (Figure 7E). We found that tTRRAP has a milder effect than full length

TRRAP in delaying cell division (Figure 7E). However, the effect on cell cycle is only partially reverted, suggesting that other domains of TRRAP may be involved in this process. Of notice, H3K9me3 was still induced by this truncated version of TRRAP (Figure 7D). This suggests that, in addition to histone acetylation, other chromatin-related functions of TRRAP may be associated with the mitotic defects.

## **Discussion**

In this work we have identified TRRAP as a novel target of the APC/C complex, whose degradation is necessary for proper chromosome segregation and mitotic exit. TRRAP is known to have different roles during the cell cycle. In the G1/S transition it is involved in the transcription of histones (14). Later, during S phase, it participates in the repair of damage related with DNA replication and in the transcription of SAC proteins (12). The present report may explain how and why after these fundamental roles, TRRAP degradation becomes necessary at the G2/M transition.

The APC/C complex is considered as the master regulator of the mitotic transition of the cell cycle. Specifically, by forming a complex with the Cdc20 activator, the APC/C complex induces the Dbox-dependent ubiquitination of a specific set of proteins to promote the exit from mitosis. Most of these targets have defined roles in regulating mitotic progression, including Cyclin B, Polo-like kinase, Aurora A, and Securin (15). Perhaps unexpectedly, we have identified one chromatin modifier as a direct target of the APC/C complex. In contrast, targeting the main ubiquitin ligase involved in the G1/S transition (i.e. SCF/Skp2 complex) did not have any effect on the expression of TRRAP protein (data not shown). While

functional studies are needed to fully elucidate the biological role of TRRAP degradation prior to mitosis, our data indicate that HAT-related targets of the APC/C could be destroyed early in mitosis to inactivate HATs activities and assure chromatin compaction. It is interesting to note that in comparison to other APC/Cdc20 targets, TRRAP degradation starts relatively early (i.e. around one hour before Cyclin A and Cyclin B1 destruction). Thus, together with Sgo1 (17) and p21 (18), TRRAP may be considered as one of the early targets of the APC/Cdc20 complex.

We found that although TRRAP contains a consensus D-box motif, it is not necessary for APC/C recognition and further proteolysis. Although similar destruction boxes are found in Cyclin A, Cyclin B1, Sgo1, p21 or Securin (4), there is remarkable variation between the primary destruction motifs of APC/C substrates. For example, for their APC/C-dependent degradation Nek2A has a KEN-box and a novel destruction motif at its C terminus (19), HoxC10 contains two D-boxes (20), while p21 destruction requires only a minimal Dbox (18). On the other hand, mutating the N-terminal D-box of cyclin A does not prevent its destruction by APC/C-Cdc20 (16). Similar to cyclin A, the presence of other less conserved destruction motifs may be important for Cdc20- and Cdh1-mediated proteolysis of TRRAP.

Previous studies have suggested a role for the APC/C in regulating the activity of HAT components. Specifically, two APC/C components, APC5 and APC7, were shown to interact directly with the coactivators CBP and p300, stimulating intrinsic CBP/p300 acetyltransferase activity and CBP/p300-dependent transcription (21). On the other hand, gene ablation of CBP by RNA-mediated interference markedly reduced the E3 ubiquitin ligase activity of the APC/C and the progression of cells through mitosis (21). In addition, it was shown in yeast that the APC/C is involved in the control of histone H3 phosphorylation

and the global deacetylation of H2B, H3, and H4 during exit from the cell cycle into G0 (22). Although these previous reports pointed at a role for the APC/C in regulating the physiological status of histone modifications, TRRAP is one of the first targets of the APC/C directly involved in histone modifications. Of notice, the histone methyltransferase SET8/PR-SET7 has also been shown to be an APC target (23). In addition, there are HATs with well-described mechanisms regulating their stability and degradation that are independent of APC/C. For instance, Tip60 is targeted for degradation by mono- and poly-ubiquitination by the Mdm2 ubiquitin ligase; however this regulation is not dependent on the cell cycle (24).

The importance of TRRAP degradation is highlighted by the multiple cell division defects associated with its overexpression or partial stabilization. It is possible that as a part of multiple HAT complexes, TRRAP is involved in the changes in histone acetylation related with mitosis. Indeed, we have shown that blocking the function of TRRAP involved in histone acetylation partially rescues the mitotic delay observed after TRRAP overexpression. In addition, as mutations in the centromeric/kinetochore components or epigenetic modifications of this structure may lead to chromosome missegregation and G<sub>2</sub>/M delay (25), similar to the phenotype associated with TRRAP stabilization (this study), further studies are needed to test a direct role of TRRAP in centromeric organization. An aberrant profile of histone modifications at the centromeres may lead to a defective kinetochore resulting in mitotic defects (26, 27). Of notice, we found that TRRAP overexpression is associated with a higher expression of a centromeric (H3K4me2) and a pericentromeric (H3K9me3) histone modification. In a similar way, TRRAP degradation seems to be important for appropriate chromosome compaction during mitosis. Consequently, we propose that APC/C-dependent

degradation of TRRAP, and TRRAP containing HATs, functions to prevent segregation defects and chromosomal instability (a summary of this model is presented in Figure 8).

This study demonstrates the role of tight control of histone modifying factors in a chromatin reconfiguration during mitosis and strengthens the importance of the APC/C complex as a master regulator of mitotic exit. The APC/C complex is shown here to be involved in the degradation of a chromatin modifier, linking the changes in chromatin with the timely destruction of cyclins necessary for cell cycle progression through mitosis. Therefore, regulation of chromatin compaction through histone modifiers could represent a second arm for APC/C orchestration of the cell cycle machinery. Further studies will unravel the importance of tight regulation of chromatin modifiers during normal and pathological conditions.

## **Materials and Methods**

### ***Cell culture and cell cycle analysis***

HeLa and 293T cancer cell lines (American Type Culture Collection) were grown in standard medium (DMEM or RPMI 1640, 10% foetal calf serum, 1% penicillin/streptomycin (Gibco), 1% sodium pyruvate and 1% glutamine). After culture at different time points, samples were harvested, suspended in PBS, fixed in 70% ethanol, and their DNA content was evaluated after propidium iodide (PI) staining, as previously described (28). Fluorescence-activated cell sorting analysis was carried out using a FACScan flow cytometer (Beckton Dickinson, San Diego, CA) and CellQuest software. In some experiments cells were synchronized using a double-thymidine block protocol before cell cycle analysis. In brief, 2 mM thymidine was added to culture plates with less than 50% confluence for 17 hours. Cells were released from

first block by washing and replacing with fresh medium. After 8 hours, cells were exposed again to 2 mM thymidine, and released 15 hours later by washing and replacing with fresh medium. Bromodeoxyuridine (BrdU) incorporation was used to detect cells entering and progressing through the S (DNA synthesis) phase. BrdU was added 30 min before harvesting of cells, and incorporation to the cells was assessed by flow cytometry. Simultaneous staining of DNA with PI was used in combination with BrdU, followed by two-colour flow cytometric analysis. Simultaneous staining with anti-phosphoSer10-H3 and PI was used for estimation of the mitotic index.

### ***Quantitative RT-PCR***

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Reverse transcription reactions were performed using MMLV-RT (Invitrogen) and random hexamers, on 500 ng of total RNA per reaction according to the manufacturer's protocol. Primers and probes were designed using Universal Probe Library Assay Design Center (Roche). Quantitative real-time PCR (qRT-PCR) was performed using a FastStart TaqMan Probe Master (Roche) and a MXP3000 real-time PCR system (Stratagene). Each reaction mix contained 2 µl cDNA, 1X FastStart taqMan Probe Master, 100 nM hydrolysis probe, and 200 nM of each primer. The following PCR conditions were used in triplicate wells: denaturation step at 95°C for 10 min, and 40 cycles of 95°C for 30 seconds and 60°C for 40 seconds.

### ***Immunoblotting and immunoprecipitation***

Equal amounts of protein lysates (50-100 µg) were separated by SDS-polyacrylamide gel electrophoresis on 5 or 12 % gels, and electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Primary antibodies included anti-TRRAP (Santa Cruz Biotechnology), anti-phosphoSer10-H3 (Upstate), anti-Actin (ICN Biomedicals Inc.), anti-

Cyclin B1 (Santa Cruz Biotechnology), anti-Cyclin A2 (Santa Cruz Biotechnology), anti-GFP (Sigma), anti-Flag (Sigma), anti-Cdc6 (Santa Cruz Biotechnology), anti-Cdc20 (Santa Cruz Biotechnology), anti-Cdc27 (BD Transduction Laboratories), anti-Ubiquitin (Santa Cruz Biotechnology), and anti- $\alpha$ -Tubulin (Sigma). After incubation with peroxidase-conjugated secondary antibody (Dako) protein expression was detected using ECL Western blotting reagents (Amersham Biosciences). Native cell lysis by sonication was performed as previously described (Lee & Paull, 2004). In all experiments, 1 mg of proteins was used for immunoprecipitation. Flag-Tag-TRRAP was immunoprecipitated with  $\alpha$ -Flag antibody (Sigma) and Protein A Agarose beads (Amersham Pharmacia, Piscataway, NJ).

### ***Immunofluorescence and time lapse experiments***

Cells were fixed in ice-cold methanol/acetone for 10 min, then washed and incubated with a blocking solution containing 3% BSA in 1X PBS for 1 hour. Primary antibodies anti-TRRAP, anti- $\beta$ Tubulin, anti- $\gamma$ Tubulin, anti-CENP-A, and anti-phosphoSer10-H3 were added for 2 hours, followed by 1 hour incubation with Alexa Fluor 488-conjugated secondary antibody (Invitrogen). Cells were incubated with DAPI, or To-PRO-3-iodide (Invitrogen) for 15 min before mounting using Vectashield Mounting Medium (Vector Laboratories). For time lapse experiments HeLa and 293T cells were transiently transfected with an H2b-cherry plasmid (kindly provided by P. Wachowicz, CNIO Madrid, Spain) and TRRAP expression vectors. An Axiovert LSM 510 confocal microscope (Zeiss) was used for image collection. All images were analysed using ImageJ (NIH, USA) or LSM image browser software (Zeiss).

### ***Metaphase spread***

In situ chromosome and metaphase chromosome spreads were prepared and subjected to immunofluorescence staining as described previously (29) except that the chromosomes were



treated with 0.056 M of hypotonic solution and chromosome spreads were prepared by vertically dropping the cell suspension with a Pasteur pipette onto the slide. Image analysis was performed using Image J software.

### ***Mutagenesis and Transfections***

Stratagene QuikChange XL mutagenesis kit was used to produce Dbox TRRAP mutants on a GFP-Flag-TRRAP expression vector. Primers for mutagenesis are included as Supplementary Table 1. Transient overexpression of TRRAP proteins was performed in HeLa and 293T cells by transfection of 5 µg of either construct by lipofectamine (Invitrogen). The HA-tagged Cdh1 (Addgene plasmid 11596) and HA-tagged Cdc20 (Addgene plasmid 11594) plasmids were described previously (30).

### ***RNA interference to knock-down Cdc20***

The siRNA sequences used for targeted silencing of Cdc20 and non-targeting siRNA were chosen as recommended (ON-TARGETplus SMART pool L-xxxx, Dharmacon Incorporation, Chicago, IL). Preparation of the duplexes was carried out according to the manufacturer's instructions. Transfection of siRNA duplexes was carried out using Dharmafect reagent (Dharmacon). Expression level of Cdc20 mRNA was measured 48 h and 72h after transfection.

### ***Statistical Analysis***

Means and differences of the means with 95% confidence intervals were obtained using GraphPad Prism (GraphPad Software Inc.). Two-tailed student t test was used for unpaired analysis comparing averages between classes. P values < 0.05 were considered statistically significant. ImageJ 1.46rm (National Institutes of Health, Bethesda, USA) was used for

western blot densitometry, analysis of metaphase spreads, and calculating intensity of fluorescence after transfections.

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### **Author contributions**

G.I., H.H., M.M. and MP.C. performed all the experiments. MG.F. designed the TRRAP-GFP plasmids and performed the initial ubiquitination experiments. H.H. and Z.H. coordinated the project and wrote the manuscript. All authors discussed the results and manuscript text.

### **Competing interests statement**

The authors declare no competing financial interests.

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## Figure Legends

### ***Figure 1. TRRAP expression during the cell cycle***

**A.** 293T cells were synchronized with a double thymidine block, and released for several time points. Cell cycle profiles were obtained by flow cytometry after propidium iodide (PI) staining of DNA (left panel). Protein levels corresponding to each time point are shown in the right panel for TRRAP, Cyclin B1, and Actin as loading control. **B.** HeLa cells were synchronized in a similar way. Left panel shows cell cycle profiles corresponding to protein levels in the right panel. In addition, bromodeoxyuridine (BrdU) incorporation profiles are shown, with PI in the x-axis and BrdU in the y-axis. Percentages shown for S phase correspond to the BrdU positive cells in a given time point. Mitotic index (MI) was obtained after double staining with PI and phospho-serine10-H3 (pS10H3) followed by flow cytometry analysis. **C.** HeLa cells were synchronized with double thymidine, and protein was collected up to 24 hours after release. Corresponding cell cycle profiles are shown in the upper right panel. Densitometry quantification and tendency curve for the western blot are shown in the lower right panel.

### ***Figure 2. TRRAP protein degradation by the Ubiquitin-Proteasome System***

**A.** HeLa and 293T cells were transfected with a Flag-TRRAP construct. Protein lysates were used for immunoprecipitation (IP) with anti-Flag antibody and western blot with anti-Ubiquitin and anti-Flag antibodies. Anti-MRE11 was used as a negative control. I= input, E= eluate. **B.** HeLa cells were treated with different combinations of nocodazol and the proteasome inhibitor MG132 during 12 hours. Total protein lysates were extracted for

western blot analysis of TRRAP, Cyclin B1, and Actin as loading control. **C.** HeLa cells were transfected with a GFP-TRRAP plasmid and grown in the presence or absence of MG132. Confocal microscope analysis shows an increased fluorescence for MG132 treated cells (quantification is included in the text). **D.** HeLa cells were transfected with a GFP-TRRAP plasmid as in **2C** and synchronized with a double thymidine block. Design of the experiment is shown in the upper panel. Cells were released in presence or absence of MG132 to monitor TRRAP protein expression after several time points. Ponceau staining was used to check for equal protein loading.

**Figure 3. Silencing of Cdc20 induces TRRAP accumulation**

**A.** Representation of TRRAP protein with different putative destruction boxes (RXXL) shown in black boxes. Specific putative destruction boxes for Cdc20 (Dbox) and Cdh1 (KENbox) recognition are shown in white boxes. Conservation of TRRAP Dbox consensus sequence between species and alignment with known Cdc20 targets is included in the lower panels. **B.** Cdc20 expression was interfered in 293T cells, as described in *Materials and Methods*. 48h and 72h after siRNA treatment, protein lysates were used for western blot analysis of Cdc20 and TRRAP expression (left panel). Right panel shows the expression after simultaneous Cdc20 interference and cell synchronization in G2/M (6 hours after double-thymidine block release). Actin was used as loading control in both experiments. **C.** Left panel: 293T cells were transfected with a Flag-TRRAP plasmid and IP for endogenous or Flag-TRRAP. Western blot was performed to detect interaction with Cdc20, using Cdc6 as negative control. Right panel: HeLa cells were used to detect interaction between endogenous TRRAP and Cdc20 during the cell cycle. Cells were synchronized with a 2T-block and



released for 6 or 9 h. Progressive interaction is shown for Cdc20, while no interaction is shown for Cdc27.

***Figure 4. APC/C ligases target TRRAP protein for degradation***

**A.** 293T cells were transfected with full length GFP-TRRAP or Cyclin B1 alone and in the presence of HA-tagged Cdh1, HA-tagged Cdc20 at ratios of 1:5 and 1:15 (TRRAP and each ubiquitin ligase). Forty-eight hours after transfection, cells were harvested and extracts analyzed by immunoblotting with anti-HA, anti-Cyclin B1, anti-GFP (to check for exogenous TRRAP expression) or anti-GAPDH antibodies. Left and right panels correspond to Cyclin B1 and TRRAP transfections, respectively. **B.** 293T cells were transfected with full length GFP-TRRAP alone (left panel), or in the presence of HA-tagged Cdh1 (central panel) or HA-tagged Cdc20 (right panel) at a ratio of 1:15. Forty-eight hours after transfection, cells were treated with 100 µg/mL cycloheximide (CHX), collected at the indicated time points and extracts were analyzed by immunoblotting with anti-GFP, anti-Cyclin B1, anti-HA or anti-GAPDH antibodies. **C.** densitometry analysis of the western blots shown in **B**.

Quantifications of Cyclin B1 and TRRAP were relative to GAPDH loading control in each of the transfection conditions (mock, Cdh1, and Cdc20). An exponential decay curve was fit for each of the conditions.

***Figure 5. TRRAP binds Cdh1 and Cdc20, but destruction motifs are not required for TRRAP proteolysis***

**A.** 293T cells were transfected with full length TRRAP (GFP-TRRAP), and co-transfected with empty vector, HA-tagged Cdh1 or HA-tagged Cdc20. After 48h lysates were used to immunoprecipitate with anti-GFP antibody followed by immunoblots with anti-GFP, anti-polyUbiquitin, and anti-HA (also shown for the non-IP fraction in the lower panel). **B.** 293T

cells were transfected with full length GFP-TRRAP, Dbox or DK mutants. Cells were co-transfected with empty vector, HA-tagged Cdh1 or HA-tagged Cdc20 at ratios of 1:5 and 1:15 (TRRAP and each ubiquitin ligase). Forty-eight hours after transfection, cells were harvested and extracts analyzed by immunoblotting with anti-HA (Cdh1/Cdc20), anti-GFP (TRRAP) or anti-GAPDH antibodies.

***Figure 6. Failure to degrade TRRAP induces segregation defects***

**A.** 293T cells were transfected with the indicated plasmids and synchronized with a double thymidine block. Immunofluorescence (IF) with pS10H3 antibody was used for analysis of segregation defects in 293T cells after mock or TRRAP transfection. **B.** Quantification of lagging chromosomes and chromosome bridges was performed by IF after nocodazol 30, 60, and 90 minutes after release. **C.** Alternatively, mitotic defects were quantified 9 hours after release from double-thymidine synchronization. Significant differences ( $P$  value  $< 0.05$ ) are indicated with a (\*). **D.** 293T cells were transfected with the indicated plasmids and exposed 1h to colcemid treatment before metaphase spreads (as described in *Materials and Methods*). The upper panel shows the different phenotypes used for quantification of the spreads. The lower panel represents the distribution of the different phenotypes. (\*) represents a significant  $P$  value when comparing partially separated and scattered spread phenotypes between mock-transfected and TRRAP-overexpressing cells.

***Figure 7. TRRAP-related mitotic delay is partially dependent on global histone hyperacetylation***

**A.** Chromatin fractionation was performed after mock or full length TRRAP transfection of HeLa cells. DNA bound fractions were used to study a panel of histone marks and the heterochromatin protein 1 (HP1). Total histone H3 and Actin were used as loading controls.

B. A truncated form of TRRAP (tTRRAP) was engineered by introducing two stop codons after the Dbox/KEN motifs in the full length GFP-TRRAP plasmid. The resulting protein after mutagenesis is expressed as a GFP/Flag/Dbox/KENbox containing N-terminal TRRAP of approximately 210 kDa. C. Immunoprecipitation of TRRAP and tTRRAP was done with anti-GFP antibodies to study their ubiquitination. D. Chromatin fractionation after TRRAP and tTRRAP transfection was done to study panAcH4 and H3K9me3 expression in HeLa cells. E. The same constructs were co-transfected with H2b cherry in HeLa cells, and followed in a time lapse experiment 48 hours after transfection. Nocodazol was added at 100 ng/ml during the last 16 hours. The number of successful cell division per 100 cells was counted during the 30 minutes after release from nocodazol-induced arrest.

***Figure 8. Model of TRRAP regulation during the cell cycle***

Based on previous reports and the present work, this model depicts the activities of TRRAP and its distribution during the cell cycle. TRRAP protein is low in G0/G1 and reaches maximum levels during S phase. In the G1/S transition TRRAP is involved, for example, in the transcription of histones required for chromatin synthesis. Later, during S phase, it also participates in the repair of damage related with DNA replication. Drastic reduction of TRRAP levels occurs before mitosis by a mechanism dependent on the APC/Cdc20 complex and the ubiquitin/proteasome system. A fraction of TRRAP may have a role in setting the specific pattern of centromeric histone marks required for correct segregation.

### ***Supplementary Data***

#### ***Supplementary Figure 1. TRRAP expression after Nocodazol synchronization***

HeLa cells were synchronized in prometaphase with nocodazol. Upper panel shows cell cycle profiles corresponding to protein levels in the lower panel. BrdU incorporation and pS10H3 staining was used as in Figure **1B**. A= asynchronous control cells. MI= mitotic index.

#### ***Supplementary Figure 2. Destruction motifs are not required for TRRAP proteolysis***

**A.** 293T cells were transfected with wild type (wt) or mutant Dbox Flag-TRRAP expression vectors. Anti-Flag antibody was used for immunoprecipitation, followed by immunoblot to detect interaction with Cdc20. **B.** 293T cells were transfected with wild type (wt) or mutant Dbox and Dbox/KENbox (DK) Flag-TRRAP constructs. Protein stability was checked several time points after release from nocodazol synchronization using anti-TRRAP antibody (targeting wild type and mutant proteins). **C.** HeLa cells were non-transfected or transfected with wt TRRAP or Dbox mutant. TRRAP expression was assessed in the presence or absence of 4h of proteasome inhibition with MG132, followed or not by a 24 hours release. Both endogenous and exogenous TRRAP are shown. Ponceau staining is included as protein loading control.

#### ***Supplementary Figure 3. Time lapse analysis of TRRAP-overexpressing cells***

HeLa and 293T cells were transiently transfected with an H2b-cherry plasmid with or without TRRAP expression vectors. Cells were synchronized according to the strategy shown in **A**. Comparison between mock transfected and TRRAP-transfected (GFP) mitosis is shown in **B**, with quantification of the time from metaphase plate formation. Three examples of aberrant segregation in TRRAP-expressing cells are shown in **C**. Experiment 1 shows a chromosome

bridge, while experiments 2 and 3 show a failed cytokinesis. Corresponding phase contrast images are shown for experiments 2 and 3. The 3 examples presented show a prolonged mitosis (>20 min from the time of metaphase plate formation). **D.** Time quantification of 20 mitosis is shown for TRRAP overexpressing cells. All control cells (transfected with H2b-cherry alone) completed mitosis within 15 minutes (at least 20 mitosis counted).

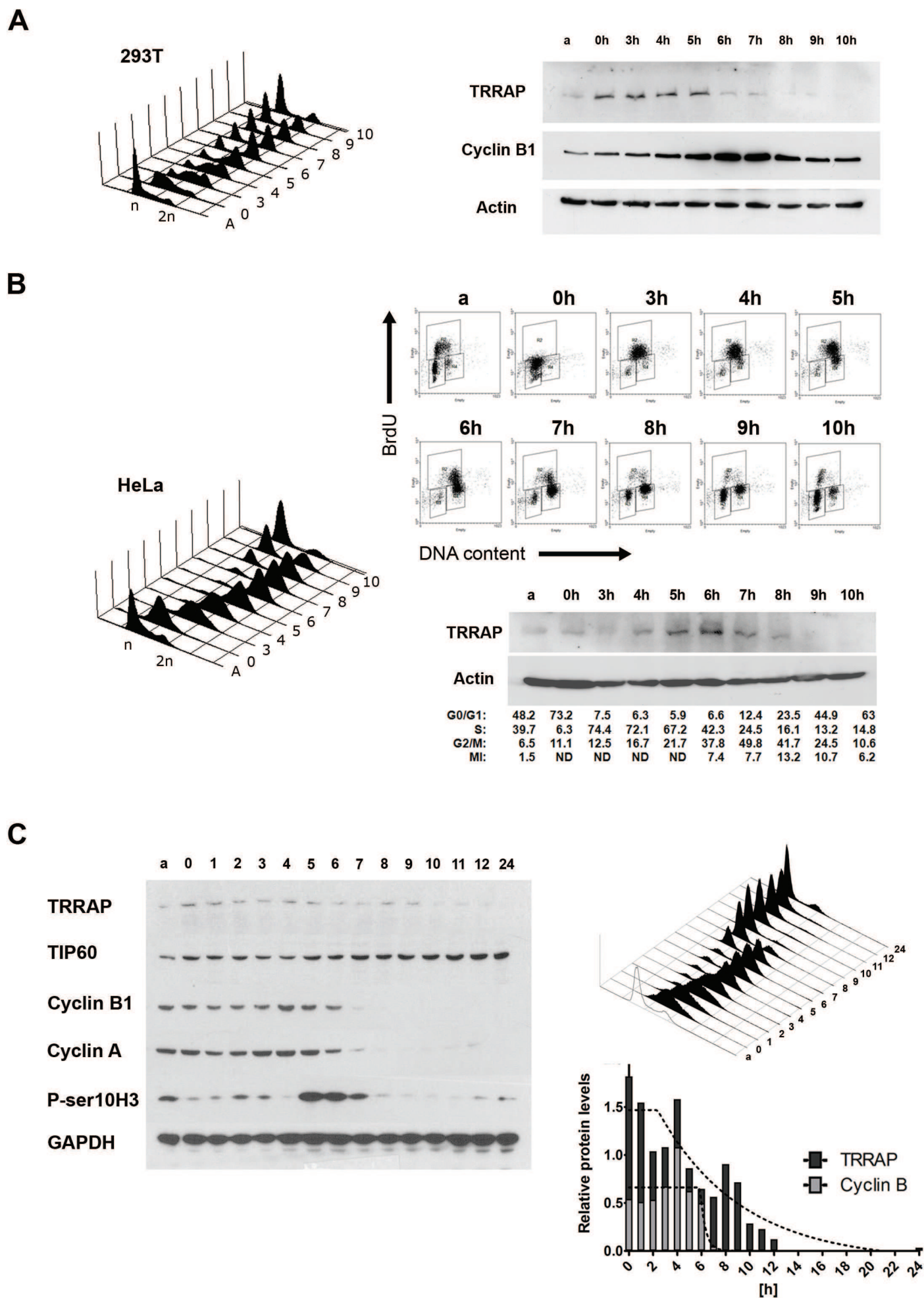
***Supplementary Table 1. Primers used for mutagenesis of TRRAP Dbox and KENbox motifs.***

1. Primers used for TRRAP-Dbox mutant

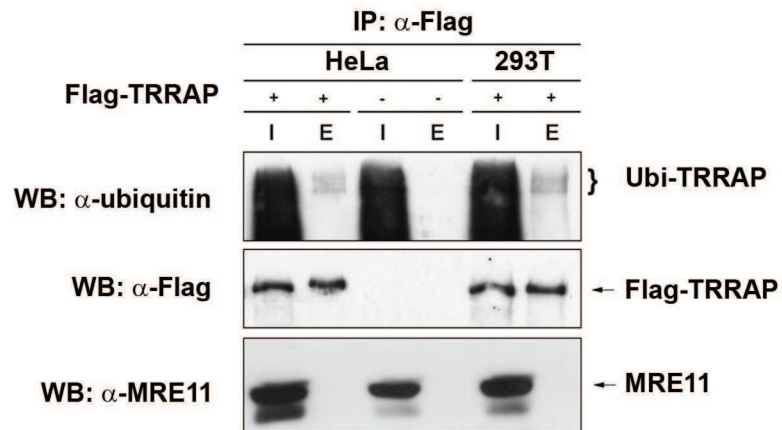
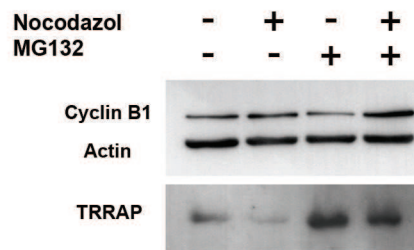
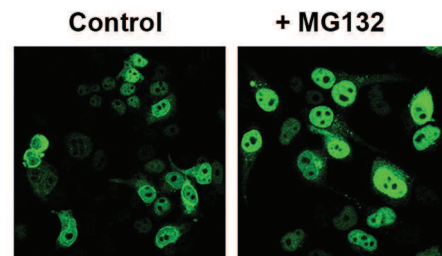
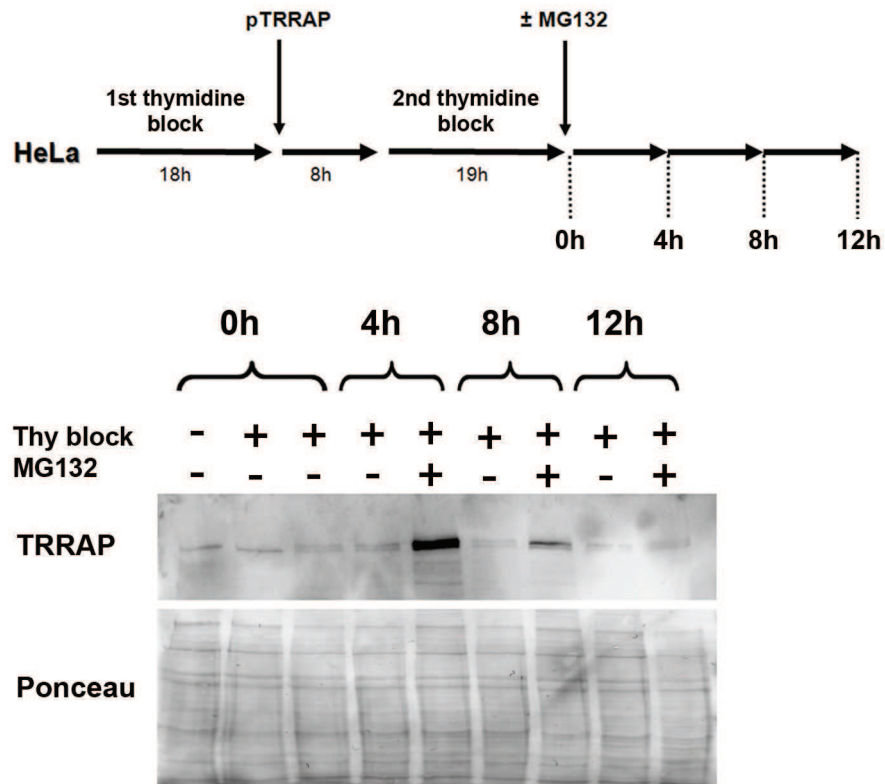
Primer Name	Primer Sequence (5' to 3')
del4956-5222	5'-gacgccagacctctgtggaaggagcccaag-3'
del4956-5222-antisense	5'-cttgggctccttcacagaggtctggcgctc

2. Primers used for TRRAP combined Dbox and KENbox mutant

Primer Name	Primer Sequence (5' to 3')
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del4956-4982-antisense	5'-cagggatgaacctcagaggtctggcgctc

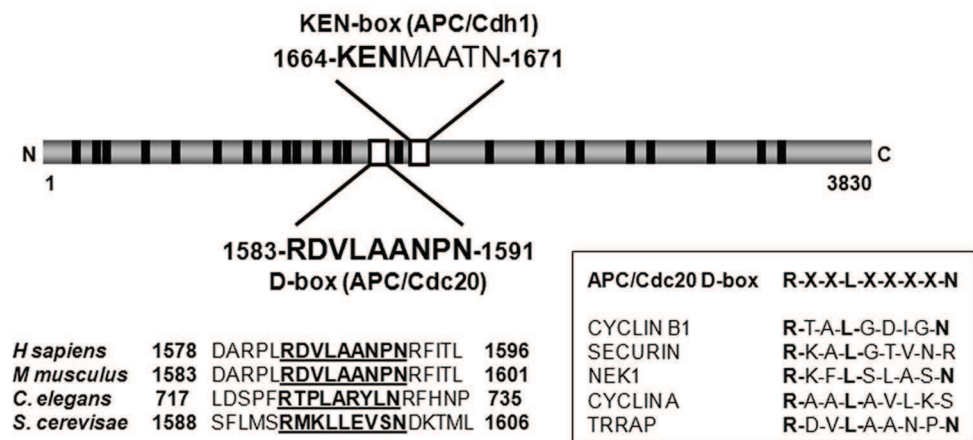


**Figure 1**

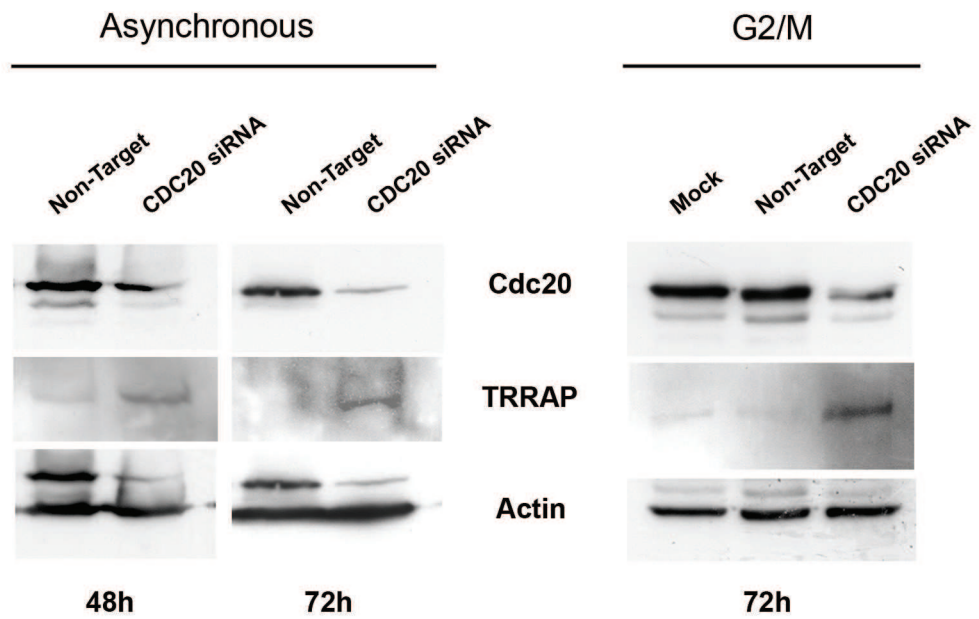
**A****B****C****D****Figure 2**



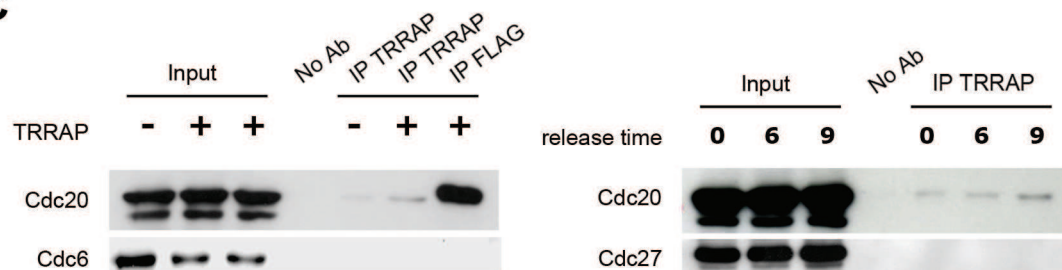
**A**



**B**

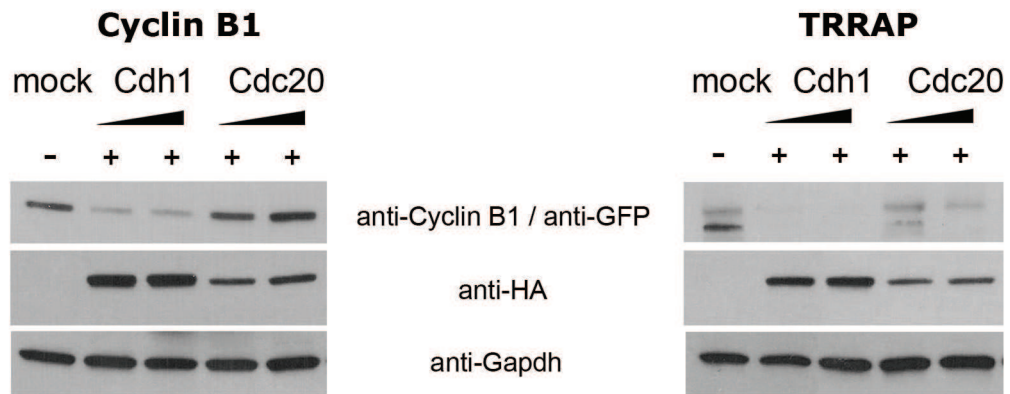
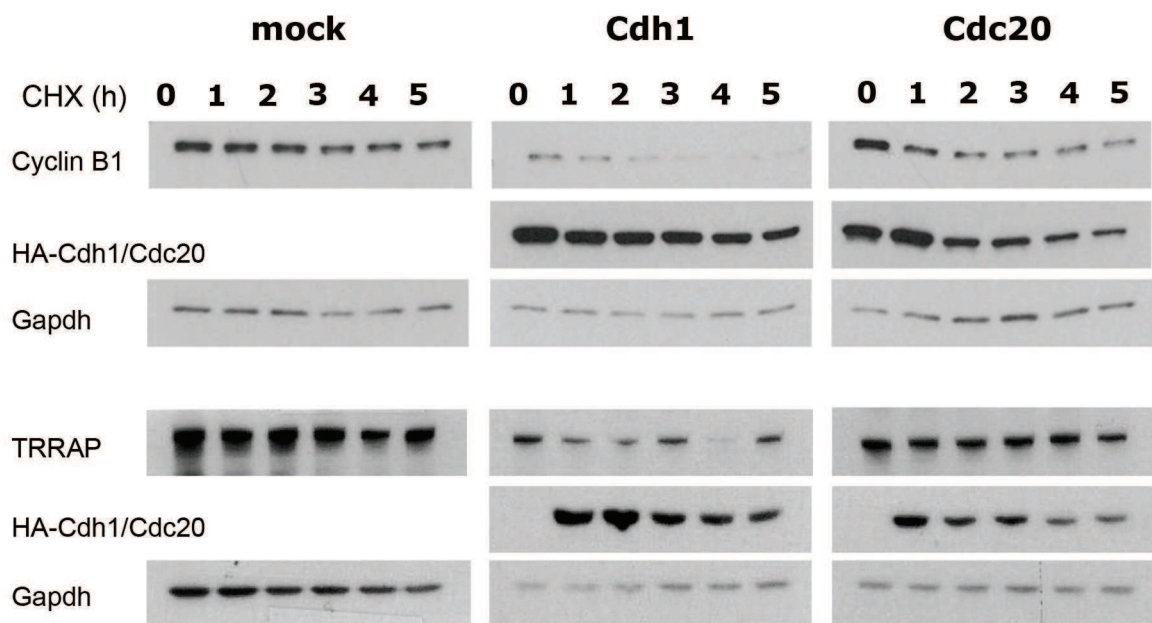
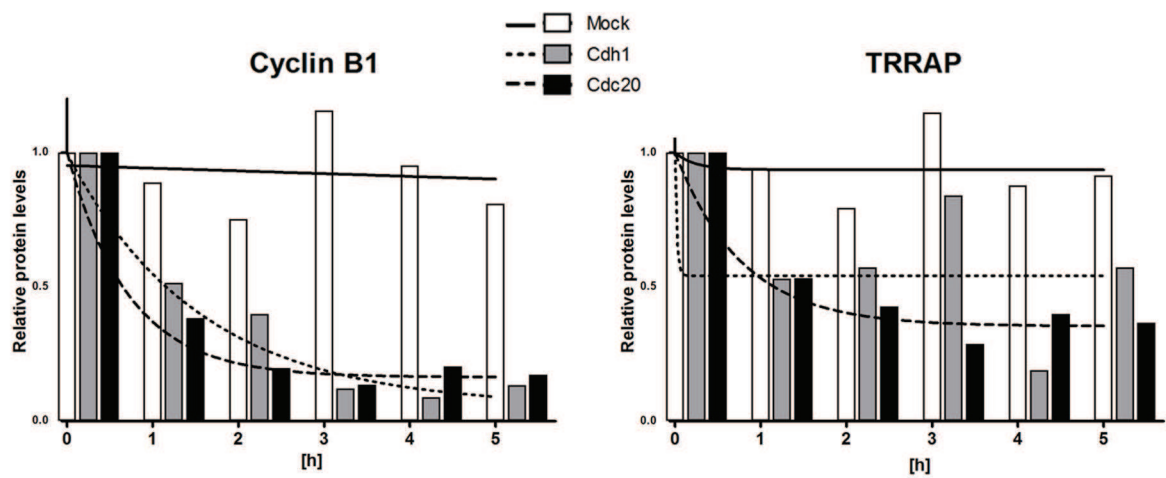


**C**



**Figure 3**



**A****B****C****Figure 4**

**A**

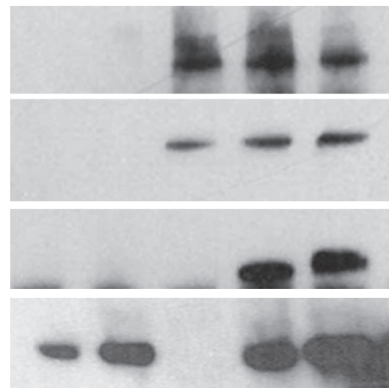
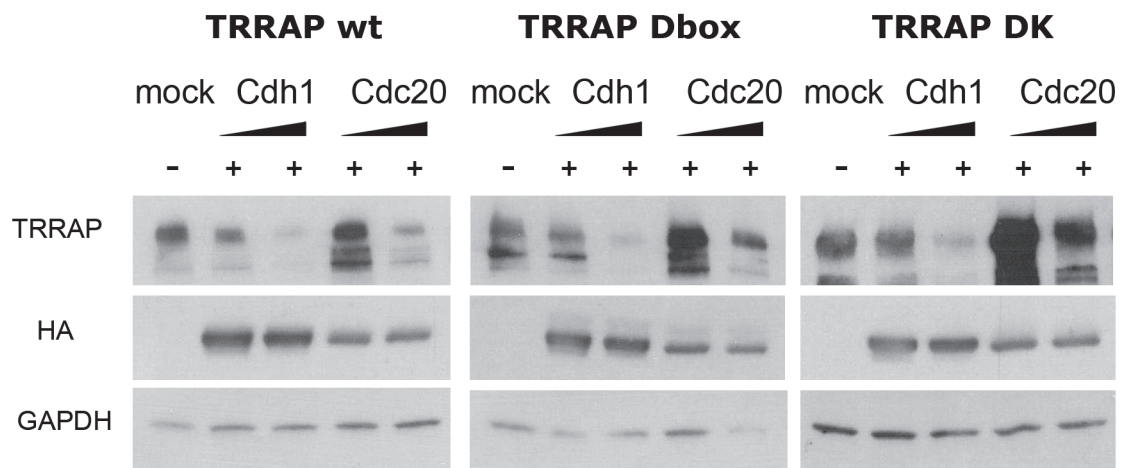
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Cdh1	-	+	-	-	+

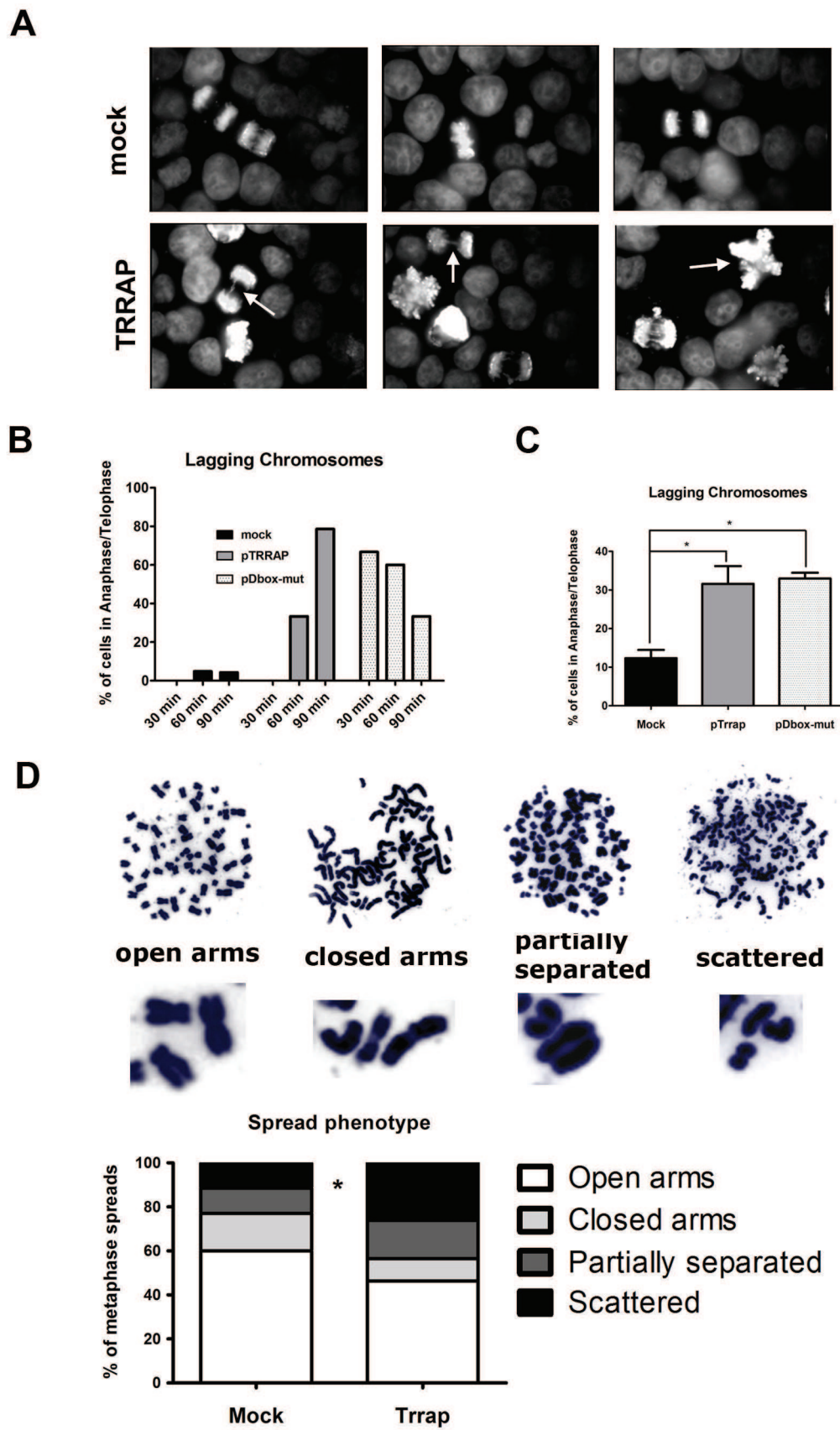
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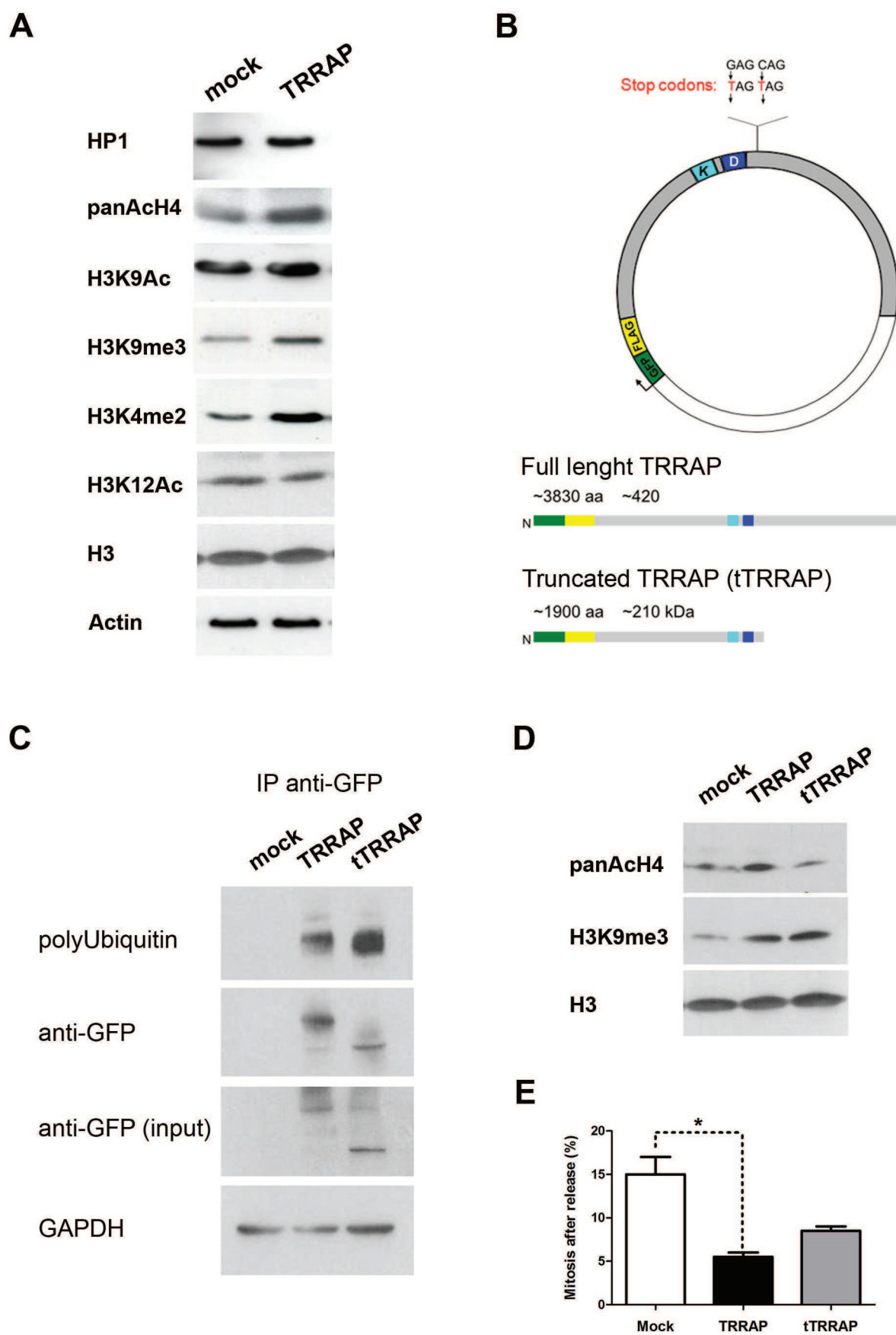
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HA (input)

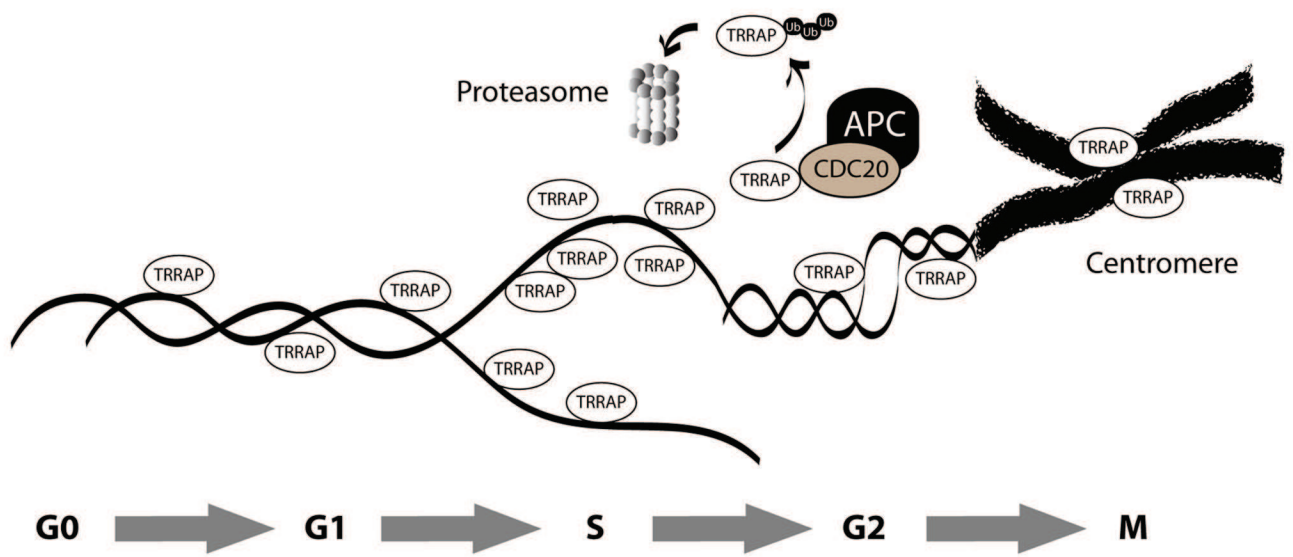
**B****Figure 5**



**Figure 6**



**Figure 7**



**Figure 8**

## 5. References

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