Université de Sherbrooke

Crosstalk between TAF6δ and Notch Signalling Pathways in Cancer Cell Lines

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Mémoire présenté à la Faculté de médecine et des sciences de la santé
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Dedicated to God
Guadalupe Virgin
Chucho Priest
My Lovely Parents and Family
et
Pour l'amour de ma vie, Mon Chéri

Don't give up, please don't give way,
Even if the cold burns,
Even if fear bites,
Even if the sun sets,
And the wind goes silent,
There is still fire in your soul
There is still life in your dreams.

Because every day is a new beginning,
Because this is the hour and the best moment.

By Mario Benedetti

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Thank you GOD for everything in my life. The good and bad. Some were blessings and some were Lessons! ^^

Some people has an American dream but in my case, I had a Canadian dream. Coming to Canada, the "Land of Endless Opportunities" and study here was my wildest, incredible and exciting dream. I made it.

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The future belongs to those who believe in the beauty of their dreams….Eleanor Roosevelt

Thanks a lot to CANADA and Université de Sherbrooke for the greatest opportunity in my life and thanks for my scholarship, tranzyme pharma bursary and the funding agencies (IRSC/CIHR, CRSNG/NSERC).

RÉSUMÉ

Interaction entre les voies de signalisation TAF6δ et Notch dans des lignées de cellules Cancéreuses

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Mémoire présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de maître ès sciences (M.Sc.) en microbiologie et d´infectiologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

La voie de signalisation de Notch contrôle de multiples processus cellulaires, telle la différenciation, la prolifération cellulaire et l'apoptose. Son activation repose sur la liaison du récepteur Notch par son ligand. Par la suite, le domaine intracellulaire actif de Notch (NIC) est relâché après son clivage médié par la γ-sécrétase. Cela permet au NIC d'être transporté au noyau où celui-ci lie la protéine CSL et active la transcription de ses gènes cibles, comme Hes1. TAF6 est une sous-unité du facteur de transcription général TFIID qui joue un rôle important dans la régulation de la transcription effectuée par l'ARN polymérase II. L'isoforme TAF6δ peut induire l'apoptose et aussi l'expression des gènes cibles de Notch. Cette étude a pour objectif d'explorer l'interaction croisée entre les voies de signalisation de Notch et de TAF6δ et leur impact sur l'apoptose. Pour valider l'impact de l'expression de TAF6δ sur la voie de signalisation de Notch, nous avons effectué une analyse par micropuce. L'expression de TAF6δ médiée par la transfection de SSOs (oligonucléotides Splice-Switching) a révélé une induction γ-sécrétase dépendante de gènes cibles de Notch dans les cellules HeLa. La cytométrie de flux a en outre montré que l'apoptose TAF6δ-dépendante est réduite par un traitement avec des inhibiteurs de gamma-sécrétase. L'analyse par immunofluorescence a révélé que TAF6δ induit la translocation de NIC-2 au noyau. Enfin, une analyse par qPCR a montré que l'expression du gène cible Notch est augmentée dans plusieurs lignées de cellules cancéreuses en réponse à l'induction TAF6δ. Nos données montrent, que la voie de signalisation de Notch est activée par TAF6δ dans plusieurs modèles de cancer et que l'interaction entre ces deux voies contribue à l'apoptose dans un modèle de cancer du col de l'utérus.

Mots-clés : TAF6δ ; voie Notch ; l'activation transcriptionnelle ; cancer du col utérin.

SUMMARY

Crosstalk between TAF6δ and Notch Signalling Pathways in Cancer Cell Lines

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Thesis presented at the Faculty of medicine and health sciences to obtain the Master degree diploma of Sciences (M.Sc.) in Microbiology and infectiology, Faculty of medicine and health sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Background: The Notch pathway controls multiple cellular processes, such as differentiation, cell proliferation and apoptosis. Its activation is based on the ligand binding to a Notch receptor after which, the Notch intracellular active domain (NIC) is released through cleavage mediated by γ-secretase. Upon cleavage, NIC translocates to the nucleus, where it binds CSL (CBF1/Su (H)/Lag-1) and activates the transcription of its target genes such as Hes1. TAF6 is a subunit of the TFIID basal transcription complex that plays an important role in the regulation of RNA polymerase II transcription. TAF6δ is a specialized isoform of TAF6 that can induce apoptosis and induces the expression of Notch target genes. This study aims to explore the potential crosstalk between TAF6δ and Notch signalling pathways and its impact on apoptosis.

Results: To validate the impact of TAF6δ expression on the Notch pathway, we performed microarray analysis. TAF6δ induction, mediated through transfection of SSOs (Splice-Switching oligonucleotides), revealed a γ-secretase–dependent induction of Notch target genes in HeLa cells. Flow cytometry analysis further showed that TAF6δ-dependent apoptosis is reduced by treatment with γ-secretase inhibitors. Immunofluorescence analysis revealed that TAF6δ induced translocation of NIC-2 to the nucleus. Finally, qPCR showed that Notch target gene expression is increased in several cancer cell lines in response to TAF6δ induction.

Conclusion: Our data show that the Notch pathway is activated by TAF6δ in several models of cancer, and that this association contributes to apoptosis in cervical cancer.

Keywords: TAF6δ; Notch pathway; Transcriptional activation; cervical cancer.

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LIST OF ABBREVIATIONS

ADAM	*A desintegrin and metalloproteinase*
AIF	*Apoptosis-Inducing Factor*
ANK	*Ankyrin repeat domain*
Apaf1	*Apoptosis Protease Activating Factor 1*
APS	*Ammonium Persulfate*
APH1	*Anterior pharynx-defective 1*
Bad	*BCL2-associated agonist o f cell death*
Bak	*BCL2-antagonist/killer 1*
Bax	*BCL2-associated X*
B-ALL	*B- cell acute lymphoblastic leukemia*
Bcl 2	*B-cell CLL/lymphoma 2*
Bcl XL	*B-cell lymphoma-extra large*
BH	*Bcl-2 Homology*
Bid	*BH3 Interacting Death Domain Agonist*
Bik	*BCL2-interacting killer*
Bim	*B-cell lymphoma 2 Interacting Mediator o f cell death*
bHLH	*Basic helix-loop-helix*
Bmf	*Bcl2 modifying factor*
Bok	*BCL2-related ovarian killer*
BSA	*Bovine Serum Albumin*
Caspase	*Cysteinyl Aspartate Specific Protease*
CC	*Cervical Cancer*
cDNA	*Complementary Deoxyribonucleic acid*
c-MYC	*V-Myc Avian Myelocytomatosis Viral Oncogene Homolog*
CO_2	*Carbon Dioxide*
Co-R	*Corepressor's*
CPEs	*Core promoter elements*
CPF	*Cleavage and Polyadenylation Specific Factor*
CS	*Calf Serum*
CSL	*CBF1/Su(H)/Lag-1*
CStF	*Cleavage Stimulation Factor*
CTD	*Carboxyl-terminal Domain*
DAPT	*N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester*
DCE	*Downstream Core Element*
DD	*Death Domains*
DED	*Death Effector Domains*
Deltex	*Deltex E3 Ubiquitin Ligase*
DIABLO	*Direct IAP-binding protein with low PI*
DISC	*Death Inducing Signalling Complex*
Dll	*Delta-like*

DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	*Dimethyl sulfoxide*
DNA	*Deoxyribonucleic acid*
DNTP's	*Deoxyribonucleotide triphosphates*
DOS	*Delta and OSM-11-like proteins*
DPE	*Downstream core Promoter Element*
DSIF	*DRB-sensitivity-inducing factor*
DSL	*Delta/Serrate/LAG-2*
DTT	*Dithiothreitol*
EDTA	*Ethylenediaminetetraacetic acid*
EGF	*Epidermal Growth Factor*
EGTA	*Ethylene glycol-bis(2-aminoethylether)-N, N, N', N'-tetraacetic acid*
ER	*Endoplasmic Reticulum*
EtBr	*Ethidium Bromide*
FADD	*Fas Associated via Death Domain*
FACS	*Fluorescence-activated cell sorting*
FBS	*Fetal Bovine Serum*
GATA	*GATA binding protein*
GO	*Gene Ontology*
GSI	*γ-secretase inhibitor*
GTFs	*General Transcription Factors*
HD	*Heterodimerization Domain*
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
Hes1	*Hairy/Enhancer of split 1*
Herp	*Hes-related repressor protein*
HEY1	*Hairy/enhancer of split with motif YRPW 1*
HFD	*Histone Fold Domain*
HRP	*Horseradish peroxydase*
HPVs	*Human Papillomaviruses*
HR-HPVs	*High-risk human papillomaviruses*
IAP	*Inhibitor of Apoptosis Protein*
Inr	*Initiator*
IMS	*Mitochondrial Intermembrane Space*
IMM	*Inner Mitochondrial Membrane*
JAG	*Jagged*
KCl	*Potassium Chloride*
KH_2PO_4	*Potassium Dihydrogen Phosphate*
Lfng	*Lunatic Fringe*
LNR	*Cysteine-rich LNR repeats*
MAML1	*Mastermind-like*
Mcl-1	*Myeloid Cell Leukemia sequence 1*
Mg	*Microgramme*
$MgCl_2$	*Magnesium chloride*
µL	*Microlitre*
mL	*Milliliter*
µM	*Micromolar*
mM	*Millimolar*

MOMP	*Mitochondrial Outer Membrane Permeabilization*
MPT	*Mitochondrial Permeability Transition*
mRNA	*Messenger Ribonucleic acid*
MTE	*Motif Ten Element*
Na_2HPO_4	*Sodium phosphate dibasic, Disodium hydrogen phosphate*
NaCl	*Sodium Chloride*
NELF	*Negative Elongation Factor*
NCR	*Cysteine response region*
NCT	*Nicastrin*
NIC	*Notch intracellular active domain*
NLS	*Nuclear Localizing Signals*
Noxa	*NADPH oxidase activator 1*
nM	*Nanomolar*
NRARP	*NOTCH-Regulated Ankyrin Repeat Protein*
NRR	*Negative Regulatory Region*
OMM	*Outer Mitochondria Membrane*
Omi/HtrA2	*Mitochondrial Serine Protease*
Opti-MEM	*Minimal Essential Medium*
PARP1	*Poly(ADP-Ribose) Polymerase 1*
pb	*Base pair*
pRB	*Retinoblastoma protein*
PBS	*Phosphate Buffered Saline*
PCD	*Programmed Cell Death*
PCR	*Polymerase Chain Reaction*
qPCR	*Real-time Polymerase Chain Reaction*
PEN2/PSENEN	*Presenilin Enhancer 2*
PEST	*proline (P), glutamine (E), serine (S) and threonine (T) residues*
PFA	*Paraformaldehyde*
PIC	*Preinitiation Complex*
PT	*Pore Transition*
P-TEFb	*Positive Transcription Elongation Factor-b*
Pre-Tα	*Pre-T-cell receptor alpha chain*
PUMA	*p53 Upregulated Modulator of Apoptosis*
P300/CBP	*CREB-binding protein*
RAM	*RBP-J kappa-associated module*
RNA	*Ribonucleic acid*
RNA pol	*RNA polymerase*
rRNA	*Ribosomal Ribonucleic acid*
rpm	*Revolutions per minute*
RT	*Reverse transcription*
RT-PCR	*Reverse transcription polymerase chain reaction*
SHARP	*SMRT/HDAC1 histone deacetylase 1*
SDS	*Sodium Dodecyl sulfate*
SDS-PAGE	*sodium dodecyl sulfate polyacrylamide gel electrophoresis*
Smac	*Second Mitochondria-derived Activator of Caspase*
SMRT	*Silencing Mediator of Retinoid and Thyroid receptors*
SS	*Splice Sites*

SSOs	*Splice Switching Oligonucleotides*
TAD	*Transactivation Domain*
TAFs	*TBP-associated factors*
TBE	*Tris/Borate/EDTA*
tBid	*Truncated BH3 Interacting Death Domain Agonist*
TBP	*TATA binding protein*
TEMED	*Tetramethylethylenediamine*
TFIID	*Transcription factor IID*
TFs	*Transcription Factors*
TNF	*Tumor Necrosis Factor*
TM	*Transmembrane domain*
tRNA	*Transfer Ribonucleic acid*
TSS	*Transcription Start Site*
…	*...*

1 INTRODUCTION

1.1 PROGRAMMED CELL DEATH

- The term “Programmed” was defined as the “exact instance in which physiological cell death occurs” (Bruce Alberts 2002). Programmed cell death (PCD) is important in different biological phenomena such as ageing, development and pathology. Therefore, in multicellular organisms the number of cells is highly regulated by controlling not only the proportion of proliferation but also the proportion of cell death, such as the loss of cells during aging and development, thus establishing a balance (Lockshin and Beaulaton 1974, Bruce Alberts 2002). Since PCD was originally discovered, several studies reported different mechanisms of cell death. Specifically, three types of cell death have been classified:

- Type I: Apoptosis, a physiological process that kill useless cells during development, but does not generate an inflammatory reaction because the cells do not release the cellular components within the surrounding tissue, are phagocytosed quickly and do not produce anti-inflammatory cytokines (Rode 2005, Elmore 2007).

- Type II: Autophagy, a catabolic process that responds to extracellular or intracellular stress and sequestrates cytosolic structures, organelles and aggregates of proteins in a membrane vesicle called the autophagosome. Autophagosomes are degraded by lysosomes (Coates et al. 2010, Ouyang et al. 2012, Fuchs and Steller 2015).

- Type III: Necrosis, a pathological process that is distinguished by the swelling of cells and organelles, which lead to damage of the plasma membrane and release of intracellular contents. Due to the rupture of the cells and the release of their contents, necrosis results in an intense inflammatory response (Choudhury et al. 2012, Fuchs and Steller 2015).

1.2 APOPTOSIS

1.2.1 Definition

The term apoptosis was first introduced by Kerr, Wyllie and Currie (Kerr et al. 1972) and is derived from the Greek language "απόπτωσις", meaning leaves falling off trees or petals dropping off flowers (Hongmei 2012). Apoptosis represents a normal physiological mechanism that allows the removal of damaged or excessive cells to balance cell division with cell death during development. Apoptosis, also known as physiological cell death, cell suicide, cell deletion and programmed cell death (PCD) plays an important role during the physiological processes of multicellular organisms, especially during embryogenesis and metamorphosis (Gewies 2003, Choudhury et al. 2012). One of the main functions of PCD is to maintain a balance in the development and maintenance of multicellular biological systems that depends on sophisticated interconnections between the cells that form the organism. Apoptosis is also tightly regulated during development when many cells are overproduced and subsequently undergo PCD. Developmental processes require a balance between the number of cells generated by proliferation and the number of cells that are killed by cell death, contributing to the tissue-specific regulatory mechanisms underlying the formation of many organs (Zhang and Herman 2002, Gewies 2003, Dlamini et al. 2004). A relevant example is the role played by apoptosis in the regulation of the immune system. Lymphocytes T matured in the thymus are responsible for destroying infected cells in the body but before they enter the bloodstream, they have to be tested to validate their reactivity against foreign antigens, but not self-antigens. Therefore, some inefficient and self-reactive lymphocytes T are subjected to cell death controls at many points during their lifespan to maintain peripheral homeostasis and prevent autoimmunity (Figure 1) (Rathmell and Thompson 2002, Dash 2015).

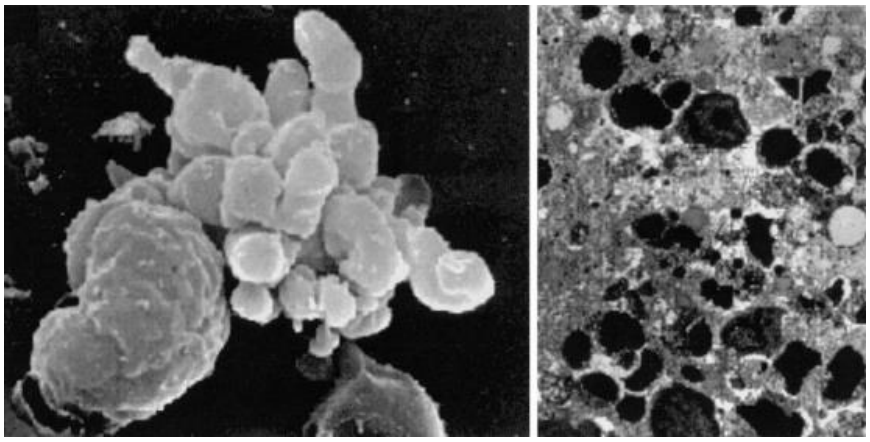
(A)  (B)

Figure 1. Apoptotic morphological changes. T-cells undergoing apoptosis *in vitro* and in the thymus after activation. Morphological appearance of the dying cell observed by electron micrographs. (A) Corresponding to a blebbing cell and (B) corresponding to nuclear condensation. (From Zimmermann, Bonzon et al. 2001).

The aberrant regulation of apoptosis is implicated in the emergence of numerous diseases, including neurodegenerative disorders (Alzheimer, Huntington and Parkinson), cardiovascular diseases (Myocardial, Stroke) and hematologic diseases (Aplasia anaemia), where there is excessive apoptosis. In contrast, insufficient apoptosis, outpaced by proliferation, can lead to cancer, restenosis, and autoimmunity (Kiechle and Zhang 2002, Reed 2002, Rajesh P. Rastogi 2009, Coates et al. 2010).

1.2.2 Morphologies of apoptosis

Apoptosis can be induced by several stimuli from outside or within the cell, such as strong DNA damage, chemical drugs or irradiation, as well as lack of survival pathways or increased death signals (Gewies 2003). The morphological hallmarks of the dying cell have been identified by light and electron microscopy, which includes membrane blebbing, chromatin condensation, nuclear DNA fragmentation, cell rounding concomitant with loss of adhesion to neighbouring cells, and cell shrinkage (Fuchs and Steller 2015). Morphological changes in cell shrinkage are visible by light microscopy, such as small size, tightly packed organelles and condensed cytoplasm. Electron microscopy has also been used to detect subcellular changes at higher resolution (Elmore 2007). Cell fragments are compacted into membrane-

bound apoptotic bodies containing organelles, cytosol and condensed chromatin that are rapidly phagocytosed by macrophages and neighbouring cells such as neoplastic and parenchymal cells. Ultimately, apoptotic bodies are degraded in phagolysosomes of phagocytic cells (Van Cruchten and Van Den Broeck 2002, Elmore 2007). All the morphological changes in apoptotic cells are caused by a number of molecular and biochemical events that includes the involvement of proteolytic enzymes that permit the cleavage of DNA into oligonucleosomal fragments and the cleavage of a multitude of protein-specific substrates, which often establish the integrity and shape of the cytoplasm or organelles (Saraste and Pulkki 2000).

1.2.3 Mechanisms of apoptosis

The mechanism of apoptosis is a specialized cascade of consecutive molecular events that have been categorized into two broad pathways: the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial pathway). Both pathways ultimately converge to activate effector caspases that are essential for the orchestrated sequence of biochemical events during programmed cell death (Choudhury et al. 2012).

1.2.3.1 The caspases

Caspases (cysteine aspartate specific proteases) are a family of cysteine proteases, which are expressed as inactive proenzymes (zymogens), also known as procaspases. Their structures can be classified into three domains: A N-terminal regulatory prodomain, the catalytic center containing the active site cysteine within a conserved pentapeptide sequence QACXG and a small C-terminal subunit (Figure 2).

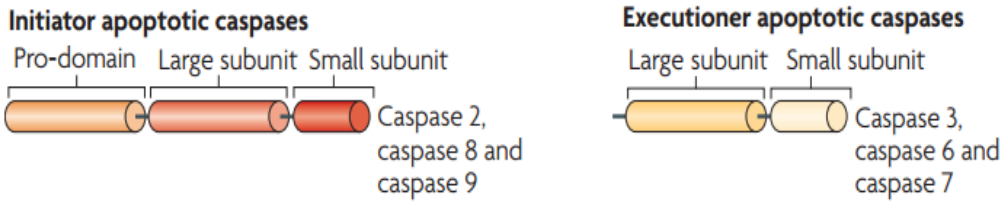


Figure 2. Classification of Caspases (Cysteine Aspartate acid proteases). Apoptotic caspases can be divided into two classes: initiator and executioner caspases. (From Tait and Green 2010).

The procaspases are activated by proteolytic cleavage at specific aspartate residues (Zimmermann et al. 2001, Choudhury et al. 2012). Upon maturation, the procaspases are proteolytically processed (aspartate cleavage site) between the large and small subunit, resulting in a small and large subunit that allows the formation of a heterotetramer composed of two small and two large subunits forming an active caspase (Figure 3) (Gewies 2003).

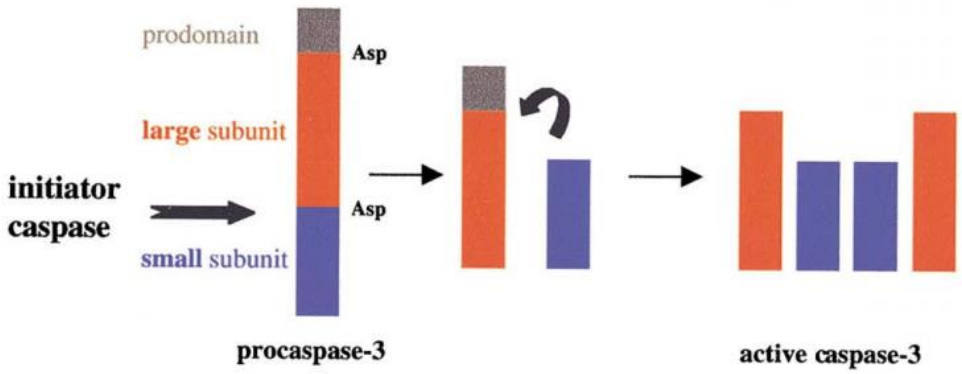


Figure 3. Activation of procaspase-3 by cleavage. Schematic processing of caspases during activation. (From Zimmermann, Bonzon et al. 2001).

In mammals, based on structure and function, caspases have been subdivided into two main categories (Figure 2). The first category is the initiator caspases, which include caspases 1, 2, 4, 5, 8, 9, 10, 11 and 12, which contain a long amino- terminal prodomain that plays a regulatory role in activating downstream effector caspases. The second category is the executioner caspases, also called effector caspases, which include caspases 3, 6, 7 and 14 contain a small prodomain and are responsible for cleavage of different cellular substrates. Caspases can be further classified into two subclasses. The first subclass is inflammatory caspases, which includes caspases 1, 4, and 5 that are involved in cytokine activation and the second subclass is other cellular caspases, including caspases 11, 12, 13 and 14, whose roles are less well established (Rajesh P. Rastogi 2009, Choudhury et al. 2012, Dipak D. Ghatage 2012, Fuchs and Steller 2015).

1.2.3.2 Extrinsic pathway

The extrinsic signalling pathway can be activated by ligation of death receptors (Fuchs and Steller 2015). These death receptors are part of the tumor necrosis factor receptor (TNF) gene superfamily (which includes, FasR, TNFR1 and DR4) and are localized on the surface of the cell then became active through binding specific ligands such as Fas ligand (also called CD95L), TNF alpha and TRAIL (also known as Apo2L). Subsequently, a conformational change exposes the death domain that allows the recruitment of adapter proteins (such as FADD or TRADD dependent on the active receptor), forming the Death Inducing Signalling complex (DISC). DISC can associate with procaspase 8, resulting in autocatalytic activation of procaspase 8. Active caspase 8 can then induce the initiation of apoptosis by cleavage and activation of effector caspases (caspases 3, 7 and 6) (Figure 4) (Gewies 2003, Elmore 2007, Choudhury et al. 2012).

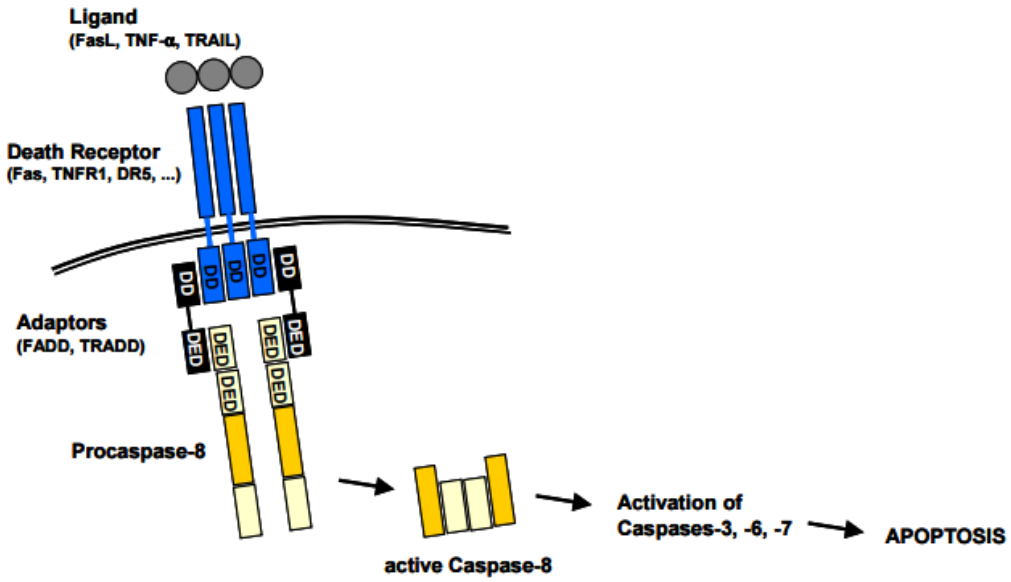


Figure 4. Receptor-mediated caspase activation at the DISC. Upon ligation, the trimeric death receptor recruits adaptor molecules via its cytoplasmic death domains (DD). Besides possessing DDs, the adaptors additionally contain death effector domains (DED) which recruit procaspase-8 to the receptor complex, which now is called the death-inducing signalling complex (DISC). Procaspase-8 is activated by autoproteolytic cleavage and forms the active caspase-8. The initiator caspase-8 cleaves and thereby activates effector caspases for the execution of apoptosis. **(**From Gewies 2003).

The crosstalk between extrinsic and intrinsic pathways can occur through caspase 8. Once caspase 8 is active, it can induce apoptosis through two parallel cascades. Firstly, it can be directly cleave and activate effector-caspases in a regular cascade. Secondly, it can mediate the cleavage of a pro-apoptotic protein Bcl-2; Bid (a BH3 domain-only protein). Truncated Bid (tBid) can then be translocated to mitochondria to induce mitochondrial outer membrane permeabilization (MOMP). MOMP causes the release of cytochrome c, which can subsequently activate caspase 9 and effector caspase 3 (Figure 5) (Tait and Green 2010, Choudhury et al. 2012).

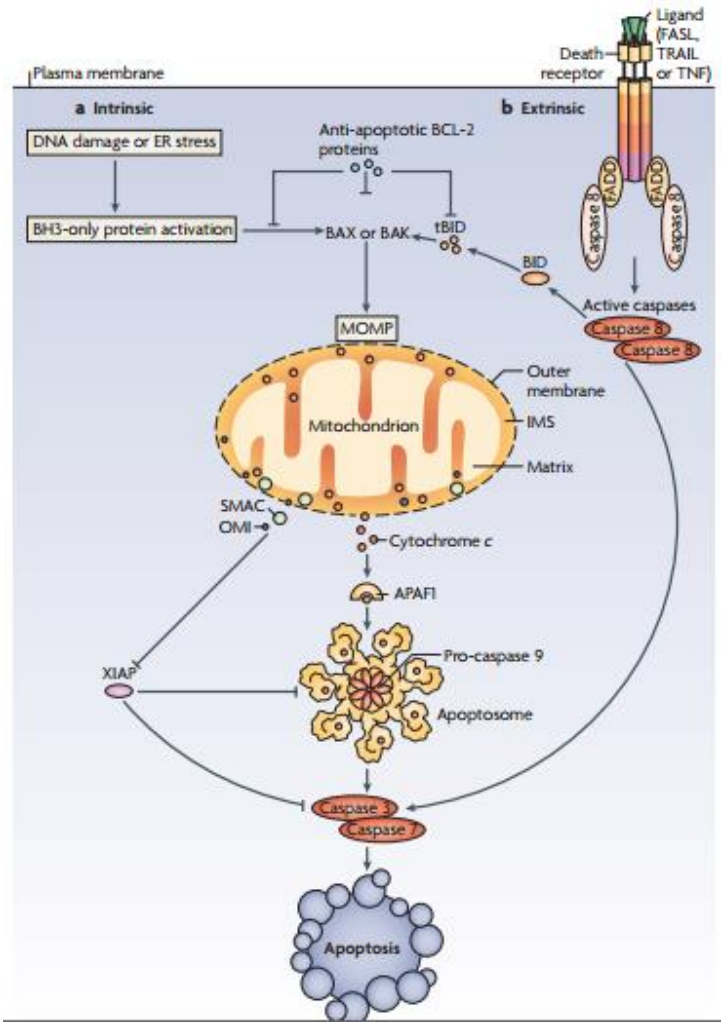


Figure 5. Intrinsic and extrinsic pathways of apoptosis. a) Intrinsic apoptotic stimuli, such as DNA damage or endoplasmic reticulum (ER) stress. b) The extrinsic apoptotic pathway is initiated by the ligation of death receptors with their cognate ligands. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BH3-interacting domain death agonist (BID), the product of which (truncated BID;

tBID) is required in some cell types for death receptor-induced apoptosis. (From Tait and Green 2010).

1.2.3.3 Intrinsic pathway

The intrinsic signalling pathway is activated by different signals coming from inside of the cell, such as strong DNA damage, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Elmore 2007, Fuchs and Steller 2015). Mitochondria play an important role in this pathway, as they are crucial to induce the caspase-cascade activation and contain pro-apoptotic proteins in the intermembrane mitochondria space (IMS), between the inner and outer membranes of the mitochondria (IMM and OMM, respectively) (Tait and Green 2010, Choudhury et al. 2012). All of the above-mentioned stimuli cause changes in the mitochondrial outer membrane permeability (MOMP). MOMP generates an opening of the mitochondrial permeability transition pore (MPT), also called PT pore resulting in a loss of the mitochondrial transmembrane potential, and stimulating the release of the sequestered pro-apoptotic proteins Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome C in the cytosol (Elmore 2007). Once the mitochondria release cytochrome c, it binds the apoptotic protease-activating factor 1 (APAF1) which allows the association of procaspase 9. When cytochrome c is associated with APAF1, a conformational change leads to oligomerization and formation of a multiprotein complex termed apoptosome. The apoptosome induces cleavage and activation of caspase 3 and caspase 7, resulting in induction of apoptosis (Tait and Green 2010, Choudhury et al. 2012). MOMP is a tightly regulated process controlled by interactions between pro- and anti-apoptotic members of the B-cell lymphoma 2 family (BCL-2) (Figure 6) (Tait and Green 2010).

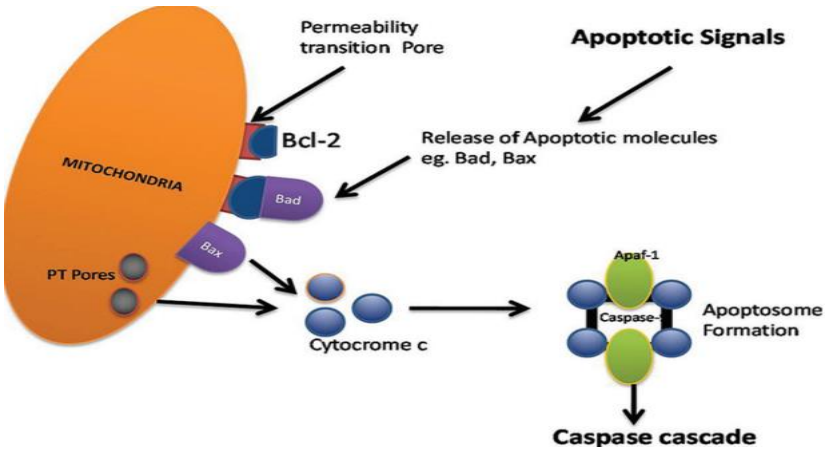


Figure 6. Intrinsic apoptosis pathway. Cytochrome c is a protein released from the mitochondria that binds to Apaf-1 and procaspase-9, in order to form the apoptosome, that then actives caspase-9 and effector caspases. (From Dipak D. Ghatage 2012).

1.2.4 Regulatory mechanisms of apoptosis

The control and regulation of apoptotic mitochondrial events are mediated by members of the Bcl-2 family proteins (Cory and Adams 2002). Tumor suppressor protein, p53 is an important pro-apoptotic factor that has a specific role in regulating the Bcl-2 family by activating the transcription of positive regulators such as DR-5 and Bax (Elmore 2007, Ouyang et al. 2012). Moreover, the PT pore formed during MOMP is due to the action of pro-apoptotic members of the Bcl-2 family, which in turn are activated by apoptotic signals such as cell stress, free radical damage or growth factor deprivation (Choudhury et al. 2012).

1.2.4.1 The BCL-2 family

The Bcl-2 family proteins were discovered as an oncogene in follicular B-cell lymphoma that inhibited cell death. This finding demonstrated for the first time that the promotion tumorigenesis is not only based on uncontrolled cell growth, but also depends on the ability to block apoptosis (Zimmermann et al. 2001, Gewies 2003). The Bcl-2 family has emerged as a critical regulator of apoptosis whose increased expression may lead to cancer and resistance to chemotherapy (Ouyang et al. 2012). The Bcl-2 family regulates apoptosis by controlling the mitochondria membrane permeability (MOMP), and members of the Bcl-2

family may possess pro-apoptotic or anti-apoptotic activity (Elmore 2007, Choudhury et al. 2012).

The Bcl-2 family is composed of 25 pro-apoptotic and anti-apoptotic members containing one or more Bcl-2 homology (BH) domains. These proteins are divided according to their function into two main categories. The first category is anti-apoptotic proteins presenting four BCL-2 homology domains including BCL-2, MCL-1, A1/Bfl-1, Bcl-B/Bcl2L10 and BCL-xL (BCL extra-large). Anti-apoptotic Bcl-2 family members prevent apoptosis by inhibiting their pro-apoptotic partners via protein-protein interactions (Zimmermann et al. 2001, Goldar et al. 2015). The second category is the pro-apoptotic Bcl-2 family proteins, which are sub-classified into two groups according to their structure. Group A, proteins having multiple BH domains (effector proteins) including: BAX, BAK, and BOK (BCL-2 related ovarian killer) and group B, proteins having only the BH3 domain, including: BID, BIM, PUMA, NOXA, BIK, BAD, HRK, and BMF (Figure 7) (Goldar et al. 2015).

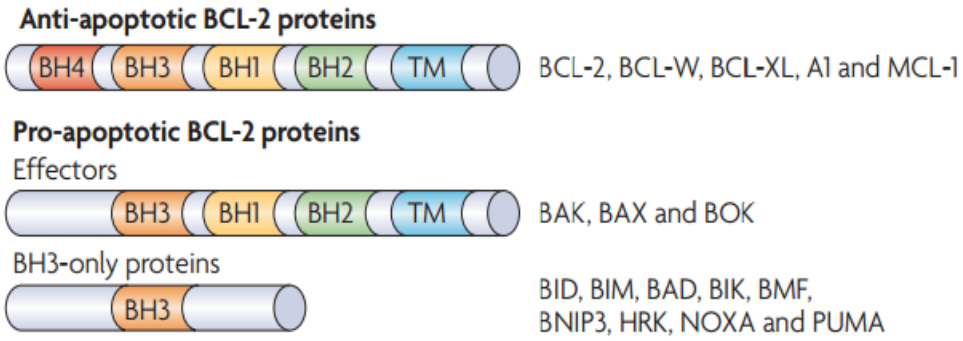


Figure 7. BCL-2 family of proteins. The B cell lymphoma 2 (BCL-2) family of proteins is divided into three groups based on their BCL-2 homology (BH) domain organization. Anti-apoptotic BCL-2 proteins and Pro-apoptotic BCL-2 proteins can be sub-divided into effectors (the proteins that actually cause mitochondrial outer membrane permeabilization (MOMP)) or BH3 only (the proteins that relay the apoptotic signal to the effectors). (From Tait and Green 2010).

Following death stimuli, pro-apoptotic proteins can undergo post-translational modifications such as dephosphorylation and subsequent cleavage that allow their activation and translocation to the mitochondria to initiate apoptosis (Ouyang et al. 2012). Therefore, pro-apoptotic BH3-only proteins act as a sensor of these apoptotic signals that permit the

activation of multidomain proteins like Bax and Bak which subsequently perform a pore (PT) on the outer membrane of mitochondria that allow the release of cytochrome c and other mitochondrial proteins. These proteins are apoptosis-inducing factor (AIF), endonuclease G, Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) and the serine protease Omi/HtrA2 (Braun et al. 2013, Goldar et al. 2015). Once these mitochondrial proteins are released into the cytosol, they can induce the caspase-cascade activation through repression of the inhibitor of Apoptosis proteins (IAP) in the case of Smac/DIABLO and Omi/HtrA2 (Gustafsson and Gottlieb 2008, Vande Walle et al. 2008). In addition, AIF and endonuclease G may also cause cell death in a caspase-dependent manner or in a caspase-independent manner based on the cellular context (Arnoult et al. 2003, Prabhu et al. 2013).

Another important pro-apoptotic BH3-only protein is Bim, well known as an efficient killer that can potentially induce cell death. Bim is essential for initiating intrinsic apoptosis pathway by apoptotic signals such as cytokine deprivation. These signals allow Bim to interact with all the anti-apoptotic Bcl-2 proteins (Mcl-1, Bcl-2, Bcl-xL, Bcl-w, and Bfl-1) and act in association with other partners like Noxa that selectively binds Mcl-1 and A1. Thus, a combination of selective binders and broader binders (Bim, Puma and tBid) promote apoptosis (Adams and Cory 2007, Sionov et al. 2015).

In summary, apoptosis can be induced by numerous apoptotic stimuli through the intrinsic pathway by intracellular signals (eg. DNA damage), or through the extrinsic pathway by extracellular signals (eg. death ligands). The intrinsic pathway can be activated by death receptors that subsequently form the DISC (Death Inducing Signalling Complex) leading to the activation of caspase 8 that can cleave and activate caspase-effectors (Caspases 3, 6 and 7) for induction of apoptosis. Simultaneously, caspase 8 can promote the activation of the intrinsic pathway through cleavage of a pro-apoptotic protein Bcl-2; Bid (tBid) which is subsequently translocated to the mitochondria to activate MOMP (strictly regulated by interactions between pro- and anti-apoptotic members of the BCL-2 family). Activation of MOMP in turn causes the release of cytochrome c into the cytosol to form the apoptosome in association with APAF1 that activates caspase 9 and effector-caspases.

Other cell signalling pathways have been reported to impinge on the core apoptotic machinery to modulate apoptosis. In relation to my research project, it has been shown that the Notch signalling pathway has an impact on cell death decisions (Zweidler-McKay et al. 2005, Robert-Moreno et al. 2007). An example of a mechanistic link between the Notch pathway and apoptosis is the known Notch target gene, Hes1. Once activated by the Notch pathway, it can regulate apoptotic signals by interacting with the PARP1 protein, causing the permeabilization of the outer membrane mitochondria (MOMP) and the release of AIF (apoptosis inducing factor), resulting in activation of the caspase-cascade and subsequent cell death (Cande et al. 2002, Kannan et al. 2011, Prabhu et al. 2013). Another example of Notch signalling to promote apoptosis includes Notch signalling reducing the transcription of the pro-apoptotic Bcl-2 family member, Bcl-xL, thereby enhancing apoptosis (Robert-Moreno et al. 2007). The regulation of Notch receptors can also modulate apoptosis by inducing pro-apoptotic BH3-only proteins, Bim and Noxa (Nickoloff et al. 2005, Konishi et al. 2010).

1.3 THE NOTCH SIGNALLING PATHWAY

More than 100 years ago, John S. Dexter discovered in *Drosophila* an irregular notched shape in the wings (Figure 8) (Dexter 1914). Later, in 1917, Morgan identified the alleles responsible for the notched wing phenotype for which he received the Nobel Prize in 1933 (Morgan 1917). Decades later, the gene was cloned in 1985 by Spyros Artavanis-Tsakonas and Michael Young, and the sequence was shown to be a cell surface receptor (Wharton et al. 1985). Furthermore, Artavanis-Tsakonas and Young characterized Notch as a regulator of cell-fate decisions (Artavanis-Tsakonas et al. 1999). Notch signalling has been shown to control several key cellular processes such as cell proliferation, differentiation, and apoptosis (Yao et al. 2007, Melino et al. 2008, Schwanbeck et al. 2011, Li et al. 2014).

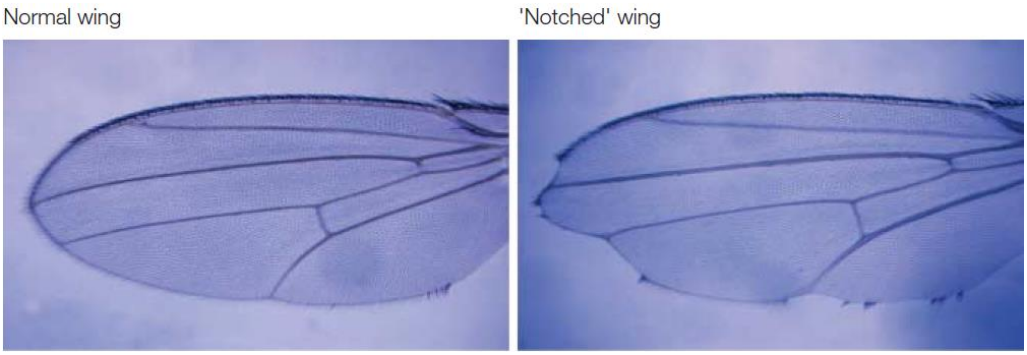


Figure 8. *Drosophila* with Notches. Wing blade of a wild-type *Drosophila melanogaster* (left), and of a mutant with a partial loss of the NOTCH gene (right). The notches, which are absent in the wild type, but clearly visible at the border of the wing blade, have given the name to the implicated gene (Notch). (From Radtke and Raj 2003).

1.3.1 Molecular biology of Notch Receptors

The Notch family of genes is conserved evolutionarily among the species. In mammals, this pathway involves a group of four receptors called Notch (Notch1-4), while *Drosophila melanogaster* has one and *Caenorhabditis elegans* has two. Each receptor is a single-pass type I transmembrane protein and is composed of two domains (Figure 9) (Kopan and Ilagan 2009, Pancewicz and Nicot 2011):

- The extracellular domain: contain different repeats that share homology with epidermal growth factor (EGF). Notch1 and Notch2 have 36 EGF repeats and it has been reported that repeat 11 and 12 are important for ligand binding. Notch 3 and Notch 4 contain 34 and 29 repeats, respectively. The number of EGF repeats and their ability to bind to calcium ions play an important role in the affinity for Notch ligands (Kopan and Ilagan 2009, Ntziachristos et al. 2014).

- The intracellular domain has several domains: RBPJ-k association module (RAM domain) and seven ankyrin repeats (ANK domain). Both domains are important for interacting with co-activators and forming the ternary complex with the mammalian CBF1/*Drosophila* Suppressor of Hairless/*C. elegans* LAG-1 protein (CSL; also known as RBPJ-k). Proline/glutamic acid/serine/threonine-rich, PEST domain

provides stability and is responsible for targeting the Notch intracellular domain (NIC) for degradation upon sel10 ubiquitin ligase recognition. Two nuclear localization sequences (NLS). The transcription transactivation domain (TAD domain), that is responsible for the activation of transcription, is strong in Notch1, weak in Notch2 and absent in Notch 3 and Notch 4 (Radtke and Raj 2003, Kato 2011).

Notch receptors also have a heterodimerization domain (HD) and a negative regulatory region (NRR) that prevents activation of the receptor in absence of the ligand (Kopan and Ilagan 2009, Wang 2011).

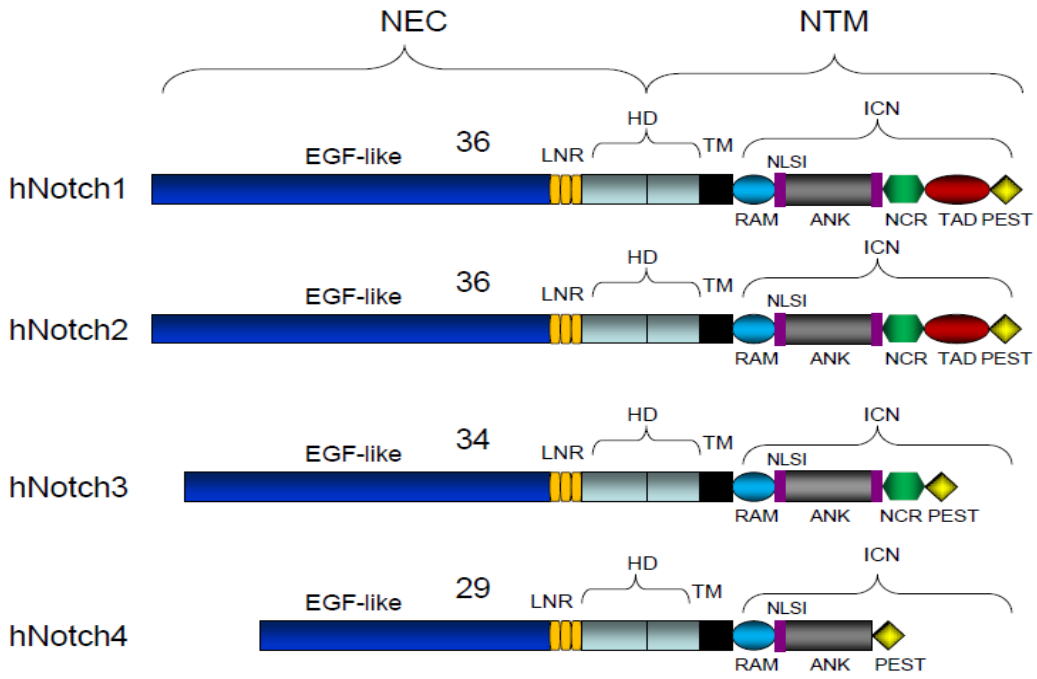


Figure 9. Structure of the four human Notch receptors. NEC: extracellular domain; NTM: transmembrane domain; EGF: epidermal growth factor; HD: heterodimerization domain; NIC: Notch intracellular domain; LNR: cysteine-rich LNR repeats; TM: transmembrane domain; RAM: RBPjk-association module; NLS: nuclear localizing signals; ANK: ankyrin repeat domain; NCR: cysteine response region; TAD: transactivation domain; PEST: region rich in proline (P), glutamine (E), serine (S) and threonine (T) residues. (From Pancewicz and Nicot 2011).

1.3.2 Molecular biology of Notch Ligands

Notch ligands are also transmembrane type I cell surface proteins. In mammalians, five Notch ligands have been reported which are classified into two families: Jagged family (JAG1-2) and Delta-like family (Delta-like 1, 3 or 4), based on the structural homology of the two *Drosophila* ligands, Serrate and Delta, respectively (D'Souza et al. 2008). Canonical Notch ligands possess a DSL domain (Delta/Serrate/LAG-2) and multiple EGF-like repeats but only the Jagged family and Dll-1 contain a DOS domain (Delta and OSM-11-like proteins). Both DSL and DOS domains are very important for receptor binding. In addition, members of the Jagged family have a cysteine-rich domain that, along with the DOS domain contribute to the structural diversity between the ligands (Figure 10) (Kume 2009).

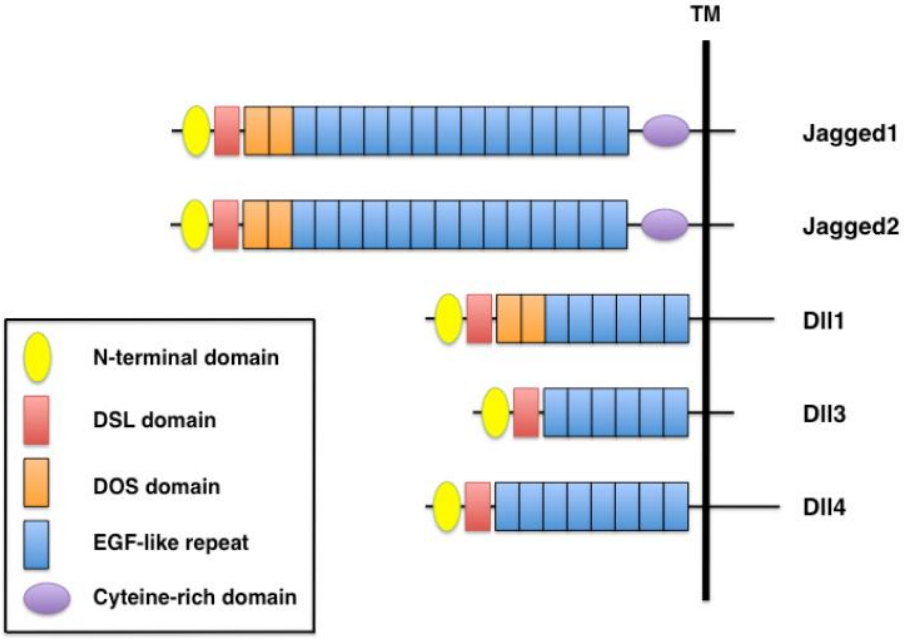


Figure 10. Domain organization of mammalian Notch ligands. All Notch ligands have an N-terminal domain, a DSL (Delta/Serrate/LAG-2) domain and EGF-like repeats. Jagged1 and Jagged2 contain a cysteine rich domain, whereas Jagged1, Jagged2, and Dll1 have two DOS (Delta and OSM-11-like proteins) domains located immediately following the DSL domain. (From Kume 2009).

1.3.3 Activation mechanism of Notch signalling pathway

Initially, Notch receptors are synthesized as a single precursor in the Trans-Golgi, where they become a non-covalently linked heterodimer as a consequence of cleavage by a furin-like convertase at the S1 site (Schwanbeck et al. 2011, Ntziachristos et al. 2014). Subsequently, the receptor becomes glycosylated by O-fucosyltransferase and Fringe Family N-acetylglucosaminidyl transferases. Following cleavage at S1 and glycosylation, the matured heterodimer Notch receptor is translocated to the cell surface (Kato 2011, Previs et al. 2015). Once on the cell surface, the activation of the Notch receptor depends on its interaction with one of its five canonical Notch ligands (JAG1, JAG2 and Delta-like 1, 3 or 4) (Kopan and Ilagan 2009, Wang 2011) in a neighbor cell. The interaction of Notch with its ligands initiates the signalling cascade by induction of a conformational change that allows proteolytic cleavage by the ADAM17 metalloprotease/TNFα converting enzyme (TACE) at the S2 site and subsequently endocytosis of the extracellular Notch domain in the signal-sending cell (Fortini 2009, Schwanbeck et al. 2011). Next, release of the intracellular active domain (NIC) is triggered by a third sequential proteolytic cleavage mediated by presenilin, the catalytic subunit of the γ-secretase complex, at the S3 site (Schroeter et al. 1998, De Strooper et al. 1999, Okochi et al. 2002). The γ-secretase complex is composed of 4 subunits: Presenilin 1/2, nicastrin (NCT), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1)) (Schroeter et al. 1998, De Strooper et al. 1999, Okochi et al. 2002, Ranganathan et al. 2011). Different compounds known as γ-secretase inhibitors (GSIs), classified into two types, can target γ -secretase cleavage at S3 pharmacologically. The two types are transition and non-transition state inhibitors. Treatment with GSI blocks the release of NIC from the plasma membrane, blocking the activation of Notch signalling (Ranganathan et al. 2011, Olsauskas-Kuprys et al. 2013). Once released, NIC is translocated to the nucleus where it acts as a transcriptional activator that interacts with the DNA-binding protein CSL and recruits co-activators such as mastermind-like (MAML1) and p300/CBP (CREB-binding protein) thereby regulating the transcription of their Notch target genes (Figure 11) (Kovall 2008, Andersson et al. 2011, Bray 2016).

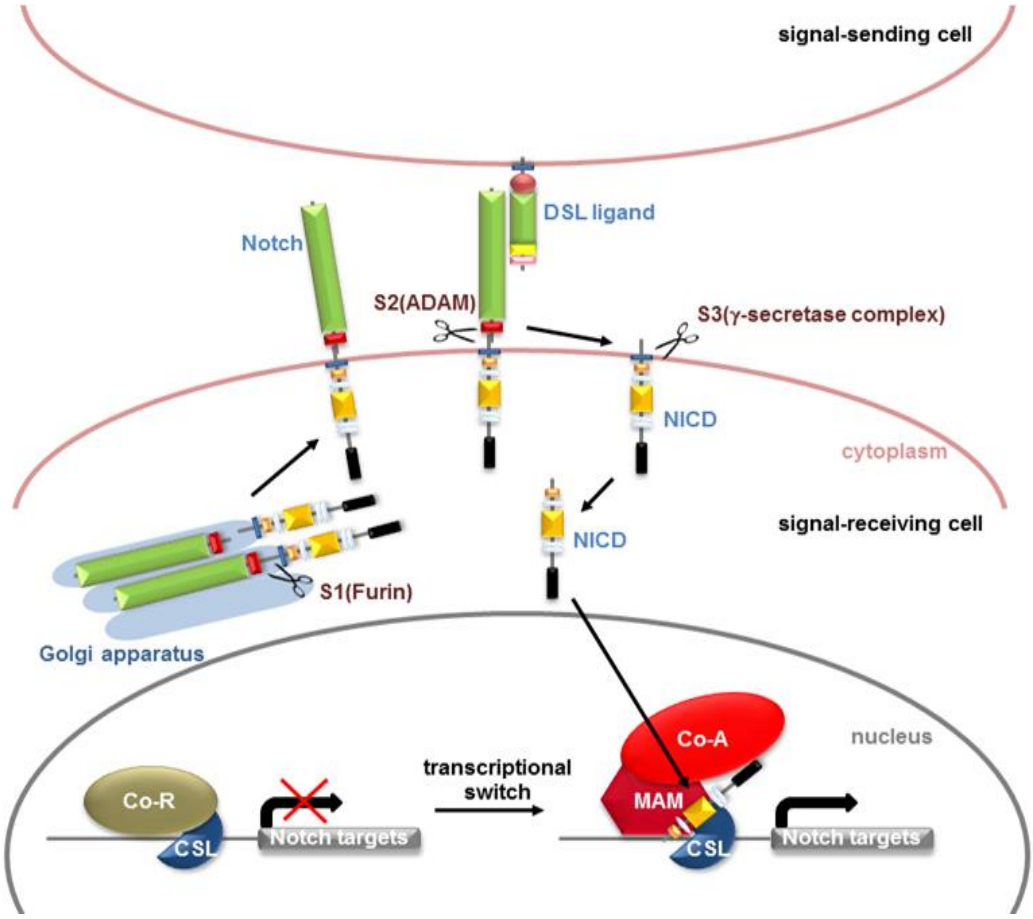


Figure 11. The canonical Notch signalling pathway. Notch receptor is glycosylated and cleaved by Furin at site 1 (S1). The interaction between Notch receptors and ligands on neighbouring cells results in the conformational change of receptor and the site 2 (S2) is cleaved by ADAM metalloproteases. Then, γ-secretase complex-mediated cleavage at site 3 (S3) releases the Notch intracellular domain (NIC). NIC then translocates into the nucleus and binds to DNA binding protein CBF1/Su (H)/Lag-1 (CSL). Transcriptional co-activator Mastermind (MAM) recognizes the NIC/CSL complex. Ternary complex formation causes the release of co-repressor's (Co-R) and recruit additional co-activators (Co-A) to activate transcription of target genes. (From Kato 2011).

In the absence of NIC, CSL acts as a transcriptional repressor through interactions with corepressors (Co-R). The Co-R including SMRT (silencing mediator of retinoid and thyroid receptors), SKIP (Ski-interacting protein), CtBP (C-terminal binding protein), Groucho/TLE (Transducin-like enhancer of split), CIR (CBF1-interacting corepressor) and SHARP (SMRT/HDAC1 (histone deacetylase 1) associated repressor protein (Figure 12) (Zhou et al. 2000, Lai 2002, Jennings and Ish-Horowicz 2008, Fortini 2009).

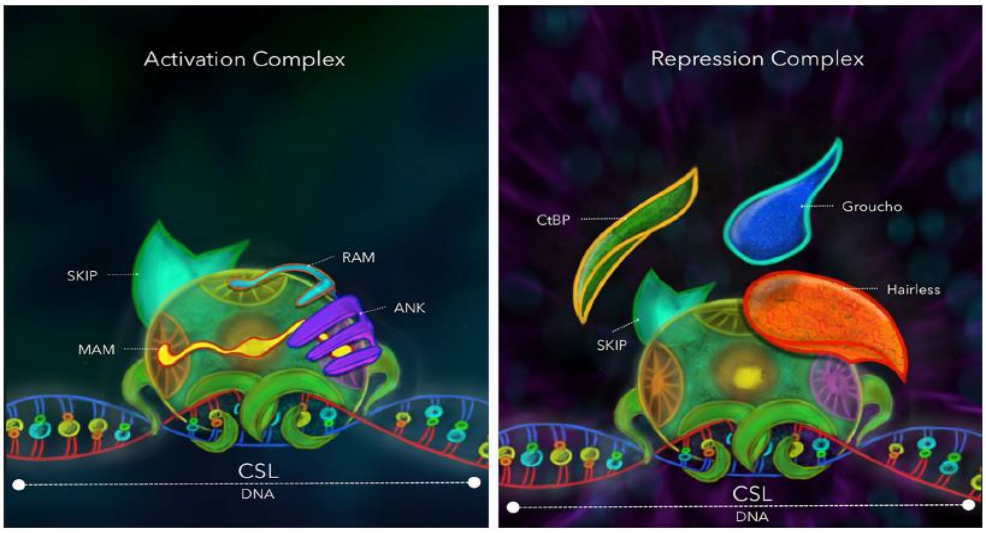


Figure 12. Comparative view of the repression and activation complexes. *Drosophila melanogaster*. The CSL transcription factor acts as a bridging protein between the DNA and a complex of proteins intended to modify chromatin topology in a specific locus. CSL: CBF1/*Drosophila* Suppressor of Hairless/*C. elegans* LAG-1 protein; RAM: RBPjk-association module of NIC; ANK: ankyrin repeat domain of NIC; NIC: Notch intracellular domain; MAM: Mastermind; SKIP: Ski-interacting protein; CtBP: C-terminal binding protein; Groucho/TLE: Transducin-like enhancer of split. (From Contreras-Cornejo, Saucedo-Correa et al. 2016).

1.3.4 Notch Target Genes

The transcription of the Notch target genes depends on three features: ligand-receptor interactions, cell type (which also include several genes that participate in different cellular processes like metabolism, differentiation and regulation of the cell cycle) and the activity of the transcriptional complex, CSL-NIC (Ntziachristos et al. 2014, Contreras-Cornejo et al. 2016).

The CSL-NIC complex activates the expression of Notch target genes including transcriptional factors, Hes (Hairy in mammals and E (spl) in *Drosophila*) and Herp (Hes-related repressor protein) (also known as Hey/Hesr/HRT/CHF/gridlock). Another Notch target gene includes: Cyclin D1, p21, NF-κB, pre-Tα (pre-T-cell receptor alpha chain), GATA3, NRARP, c-Myc and Deltex1 (Iso et al. 2003, Yin et al. 2010). The most well studied Notch target gene, and well known as a primary Notch effector, is HES/E (spl) which is part

of the basic helix-loop-helix (bHLH) family and binds DNA sequences as a dimer. This family of transcription factors is very important as effectors of the Notch pathway because they participate in the development of various organs (heart, skeletal muscles, pancreas) and cell types (Iso et al. 2003).

1.3.5 Notch in tumorigenesis

The contribution of the Notch signalling pathway to tumorigenesis is complex. Depending on the context, the Notch pathway has been shown to have an oncogenic or tumor suppressor effect (Ranganathan et al. 2011, Ntziachristos et al. 2014, Previs et al. 2015). The Notch signalling cascade shows an oncogenic effect in some cancers such as ovarian, prostate, nasopharyngeal, T-cell acute lymphoblastic leukemia (T-ALL), breast and sarcoma (Engin et al. 2009, Efferson et al. 2010, Wang et al. 2010, Wang et al. 2010, Chen et al. 2011, Hernandez Tejada et al. 2014). In apparent contrast, the Notch pathway also has a tumor suppressor effect on other cancers such as skin, endometrial, cervical, B-cell acute lymphoblastic leukemia (B-ALL) and lung (Sriuranpong et al. 2001, Nicolas et al. 2003, Yao et al. 2007, Dotto 2008, Kannan et al. 2011, Jonusiene et al. 2013). The role of the Notch pathway in cervical cancer remains ambiguous, as there is evidence that it can act as a tumor suppressor or as an oncogene (Maliekal et al. 2008).

1.3.6 The role of Notch pathway in cervical cancer

Cervical cancer (CC) is the fourth most common cancer in women and it is the seventh most common cancer worldwide, causing nearly 8% of all women deaths from cancer in 2012 (Jemal et al. 2011, Cancer 2012, Ferlay et al. 2015). The main risk factor for the development of cervical cancer is high-risk human papillomaviruses (HR-HPVs) infection (zur Hausen 2002). About 70% of all invasive cervical cancers are associated with the oncogenic HR-type 16 HPV (HPV16) and 18 HPV (HPV18). Fifty percent of HPV-positive cervical tumors carry HPV16, whereas HPV18 is present in approximately 10-20% (zur Hausen 1996, Khan et al. 2005, Smith et al. 2007, Guan et al. 2012, Goodman 2015). The development of cervical cancer develops through several steps that include. Firstly, HPV infects basal cells in the

cervical epithelium. Secondly, the HPV DNA is integrated into the genome of the host cell. Thirdly, there is viral persistence (more than two years). Fourthly, there is progression to the neoplastic phenotype (classified 1 to 3, taking 3 to 5 years) and fifthly, invasive carcinoma develops (range 10 to 30 years) (zur Hausen 2002, Maglennon and Doorbar 2012, Steenbergen et al. 2014, Goodman 2015).

Under normal conditions, the ectocervix is covered by a squamous epithelium and the endocervical canal is covered by a columnar epithelium. The basal layer of the endocervical epithelium contains precursor cells that have the ability to differentiate into squamous or columnar cells (Crum 2000, Allenspach et al. 2002). This region, known as the cervical transformation zone, is the site most susceptible to HPV infection, as well as the site for initiation of neoplastic transformation (Reid 1983, Doorbar et al. 2012, Lopez et al. 2012). HPVs expresses two oncoproteins, E6 and E7, which are essential for oncogenesis. The concerted action of E6 and E7 disrupts normal mechanisms of cell cycle regulation. In particular, E6 targets the p53 tumor suppressor protein, accelerating its degradation by the proteasome (Scheffner et al. 1990). The E7 oncoprotein functionally inactivates the pRb tumor suppressor by targeting to the proteasome for degradation (Dyson et al. 1989). pRb's interaction with the E2F transcription factors inhibit their transcriptional activity of genes required for S-phase progression. The E7-induced degradation of pRb results in the release of E2F, allowing the expression of S-phase genes and progression of the cell cycle (Münger et al. 2001, Moody and Laimins 2010, Ghittoni et al. 2015). Although E6 and E7 are necessary for the induction and maintenance of the transformed phenotype, they are not sufficient to induce the development of cervical cancer. It has been shown that at least one other cellular/genetic alteration is necessary for the development of cancer. Accumulation of evidence suggests that aberrant Notch signalling is a cellular event that can play a role in cervical carcinogenesis (Zagouras et al. 1995, Daniel et al. 1997, Rangarajan et al. 2001, Talora et al. 2002, Lathion et al. 2003, Weijzen et al. 2003, Talora et al. 2005, Wang et al. 2007).

In human cervical cancer, it was discovered that Notch expression is reduced in invasive and metastatic cells, suggesting that down-modulation of Notch pathway is required in the

tumorigenesis process (Talora et al. 2002, Sakamoto et al. 2012). In cervical cancer cells, it has been reported that the activation of Notch pathway results in inhibition of tumor growth through the induction of apoptosis and cell cycle arrest (Yao et al. 2007). In particular, in HeLa cells, the activation of the Notch pathway has been shown that decrease cell proliferation and induces apoptosis (Wang et al. 2007).

To conclude, apoptosis can be induced by the activation of the Notch signalling pathway following binding of a ligand, which results in cleavage of the Notch receptor by the γ-secretase complex, releasing the active intracellular domain of Notch (NIC) and induction of Hes1 expression (Yao et al. 2007, Kannan et al. 2011, Wang 2011). Interestingly, the pro-apoptotic transcription factor TAF6δ (Bell et al. 2001) in HeLa cervical carcinoma cells can increase mRNA expression levels of Notch target genes and also pro-apoptotic genes as it was shown by microarray assays using Splice-Switching Oligonucleotides (SSOs) (Wilhelm et al. 2008, Wilhelm et al. 2010). Indeed, HeLa cells were shown to undergo apoptosis when TAF6δ expression is triggered through SSOs (Wilhelm et al. 2010). The TAF6δ pathway therefore represents a possible new therapeutic target for treating human cervical cancer cells.

1.4 TRANSCRIPTION

The regulation of transcription is a very important step in the control of cell identity, differentiation, growth and development (Grunberg and Hahn 2013). The transcription machinery initially recognizes double-stranded deoxyribonucleic acid (DNA), but only one strand serves as a template for transcription. The transcription process begins when a specific enzyme known as RNA polymerase (RNA pol) binds to the DNA strand template to initiate the production of complementary RNA (ribonucleic acid). When the RNA polymerases are active on DNA they form a complex with different factors that allow the transcription of a specific gene (Clancy 2008). These complex sets of factors are referred as general transcription factors (GTFs), which have several functions such as promoter recognition, Pol recruitment, interaction with regulatory factors, DNA unwinding and transcription start site (TSS) recognition (Hahn 2004, Thomas and Chiang 2006).

In eukaryotes, there are three different classes of RNA polymerases: RNA pol I transcribes genes encoding 18S and 28S ribosomal RNAs (rRNAs) within the nucleoli. RNA pol III transcribe genes for 5S rRNA and transfer RNAs that play a role in the translation process and RNA pol II transcribing the messenger RNAs, which serve as templates for protein production, both localized at the nucleoplasm (Thomas and Chiang 2006, Clancy 2008). Since my work focused on the transcription factor TAF6δ, the focus here will be the mechanisms of transcriptional regulation by RNA pol II.

1.4.1 Transcription by RNA Pol II

Transcription by RNA pol II depends on a cascade of events, which are classified into three steps: initiation, which include the binding of activators to enhancers and the formation of the pre-initiation complex (PIC), elongation by RNA pol II and termination (Kandiah et al. 2014).

1.4.1.1 Initiation

The initiation of transcription by RNAPII requires basal transcription factors known as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Figure 13).

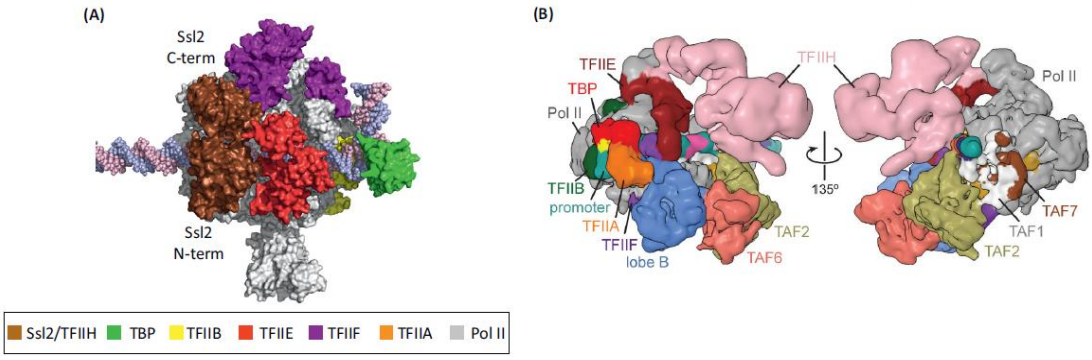


Figure 13. Architecture of the basal PIC. (A) PIC model based on crystal structures and biochemical mapping. Note that the two Ssl2 domains (labeled Ssl2 C-term and Ssl2 N-term) of TFIIE, encircling promoter DNA at positions –2 to +6 with respect to the human TSS at +1. From (Grunberg and Hahn 2013). (B) Model of the TFIID-based PIC, Cryo-EM reconstruction of the human TAF-less PIC, with fitted atomic models. (From Louder, He et al. 2016).

1.4.1.1.1 Generalities of the basal transcription factors

- TFIIA participates in transcription by stabilizing the binding between TBP (TATA-binding protein) and the TATA box by regulating the dimerization of the TBP or TFIID that accelerate DNA binding. TFIIA also plays an important role in transcriptional activation of the TATA-less promoters and RNA pol III (Hoiby et al. 2007).

- TFIIB is a factor that is associated with TBP and RNA pol II that has four functional domains: N-terminus B-ribbon, reader, linker and core (contain two cyclin-like repeats), and all of them interact with RNA pol II. B-ribbon strongly involves RNA pol II, B-reader and linker form a hairpin, termed the B-finger in the RNA pol II and B-core, in addition to bind TBP-DNA. Thus, the conformation of TFIIB within PIC plays a crucial role in transcription activation, promoter recognition and start site selection (Reese 2003, Grunberg and Hahn 2013).

- TFIID is a multi-subunit complex of mega-Dalton-sized that is thought to nucleate PIC formation on a core promoter by binding to the TATA box through its TBP subunit. TFIID also interacts with nucleosomes covalently modified and has been associated with enzymatic activities (post-translational histone modifications and transcription factors). This complex assumes a horseshoe-shaped structure containing three lobes (A, B and C). In the usual conformation, the lobe A is engaged to lobe C but the binding of TFIIA induces a conformational change that reorganizes the shape and, consequently, lobe A dislocates from lobe C to lobe B (Thomas and Chiang 2006, Grunberg and Hahn 2013, Kandiah et al. 2014).

- TFIIE, contain two TFIIEα and TFIIEβ subunits. TFIIEα containing an N-terminal WH and central Zn-ribbon, which are essential, and TFIIEβ containing tandem WH domains that are conserved. TFIIE function as a stabilizer of the non-template DNA strand and also interact directly with TFIIH because TFIIH is associated with the PIC just after TFIIE binds (Grunberg and Hahn 2013).

- TFIIF enters the PIC together with RNA pol II and contains two conserved polypeptides (Rap74/30), each containing an N-terminal dimerization domain and a C-terminal winged helix (WH). The dimerization domain binds to the lobe domain of RNA Pol II and the WH domain is bound to the protrusion of RNA Pol II (Grunberg and Hahn 2013).

- TFIIH, is a large multi-subunit complex containing ten subunits, three of them contain ATP-dependent enzymatic activities, CDK7, the Pol II CTD kinase, Rad3/XPD, a DNA helicase and Ssl2/XPB (DNA translocase). The helicase subunits and kinase enzymatic activities are required for the initiation, elongation and promoter escape steps in RNA Pol II transcription. In addition to its role in DNA unwinding, TFIIH also has a role in the phosphorylation of the RNA pol II (Reese 2003, Grunberg and Hahn 2013).

1.4.1.1.2 Mechanism of RNA Pol II initiation

These accessory factors were defined as general transcription factors (GTFs) using the following nomenclature: TF represents the Transcription Factor, the Roman numeral II indicates the transcription driven by pol II, and the "letter" corresponds to the chromatographic fraction from which the specific GTF was isolated (Thomas and Chiang 2006). The first step in the general mechanism is the recognition of the core promoter through PIC recognition of the different DNA elements located in the promoter region, known as a core promoter elements (CPEs) (Goodrich and Tjian 2010, Shandilya and Roberts 2012). These sequences are located upstream or downstream of the TSS on the target gene (Shandilya and Roberts 2012). One of the most studied CPE is the TATA box (TATAAA consensus sequence between 25 to 35 bases) upstream of the initiation site. However, not all Pol II promoters contain TATA sequences. TATA-containing promoters, which are actually the minority, account for 20-30% of the promoters in eukaryotes. In contrast, TATA-less promoters that generally direct the transcription of housekeeping genes and possess a heterogeneous TSS (Clancy 2008, Goodrich and Tjian 2010, Grunberg and Hahn 2013). The DNA sequence of the TATA box is recognized by TBP as part of the TFIID complex. TBP

possesses two domains: a highly conserved C terminus (TBPcore) that binds the TATA box and a divergent N terminus that is dispensable for viability. Once, TBP binds to the TATA sequence and induces a bend in the DNA that serves as a platform for the assembly of all GTFs and subsequent formation of PIC (Reese 2003, Shandilya and Roberts 2012). Consequently, the nucleation of PIC formation through the interactions of the TBP promoter is highly regulated (see below).

- Positive regulation involves gene-specific activator proteins (activators) that increase the binding of TBP to promoters. The activator can induce multiple genes and multiple activators can also regulate a single gene. These activators are composed of an activation domain that allows association with different transcription factors and a promoter-targeting DNA-binding domain (Bhaumik 2011, Shandilya and Roberts 2012).

- Negative regulation involves the repression of the TBP-DNA binding activity through negative factors such as Mot1/BTAF1 and NC2 (Sikorski and Buratowski 2009, Shandilya and Roberts 2012).

During PIC formation, once TBP (subunit of TFIID) recognizes and binds to the TATA box, a sequential binding of TFIIA stabilizes the interactions of the TFIID-core promoter. TFIIB interacts with TBP and DNA promoter, and is assembled to the RNA pol II-TFIIF complex. However, transcription cannot begin until TFIIB, TFIIF and RNA pol II orient the DNA template, select the TSS and then TFIIE and TFIIH will be recruited into the PIC (table 1). TFIIH possesses a helicase activity and is therefore capable of catalyzing ATP-dependent melting of the promoter and making a transition from initiation of transcription to elongation (Reese 2003, Kandiah et al. 2014). Another important element for the transition is based on the length of the transcript that has to be about 25nt in order to stable transition complex that allows the elongation process. Otherwise, transcripts with less that 5nt result in an unstable transcription complex and therefore abortive initiation (Liu et al. 2011).

Protein Complex	Functions
RNA pol II	12 Subunits; catalyzes transcription of all mRNAs and a subset of noncoding RNAs including snoRNAs and miRNAs
TFIIA	2–3 subunits; functions to counteract repressive negative cofactors like NC2; acts as a coactivator by interacting with activators and components of the basal initiation machinery
TFIIB	Single subunit; stabilizes TFIID-Promoter binding; helps in recruitment of TFIIF/Pol II to the promoter; directs accurate start site selection
TFIID	14 subunits including TBP and TBP Associated Factors (TAFs); nucleates PIC assembly either through TBP binding to TATA sequences or TAF binding to other promoter sequences; coactivator activity through direct interaction of TAFs and gene specific activators
TFIIE	2 subunits; helps recruit TFIIH to promoters; stimulates helicase and kinase activities of TFIIH; binds ssDNA and is essential for promoter melting
TFIIF	2–3 subunits; tightly associates with RNA Pol II; enhances affinity of RNA Pol II for TBP-TFIIB-promoter complex; necessary for recruitment of TFIIE/TFIIH to the PIC; helps in start site selection and promoter escape; enhances elongation efficiency
TFIIH	10 subunits; ATPase/helicase necessary for promoter opening and promoter clearance; helicase activity for transcription coupled DNA repair; kinase activity required for phosphorylation of RNA Pol II CTD; facilitates transition from initiation to elongation.

Table 1. Complexes involved in RNA Pol II PIC assembly. (From Sikorski and Buratowski 2009).

1.4.1.1.3 Mechanism of RNA Pol II elongation

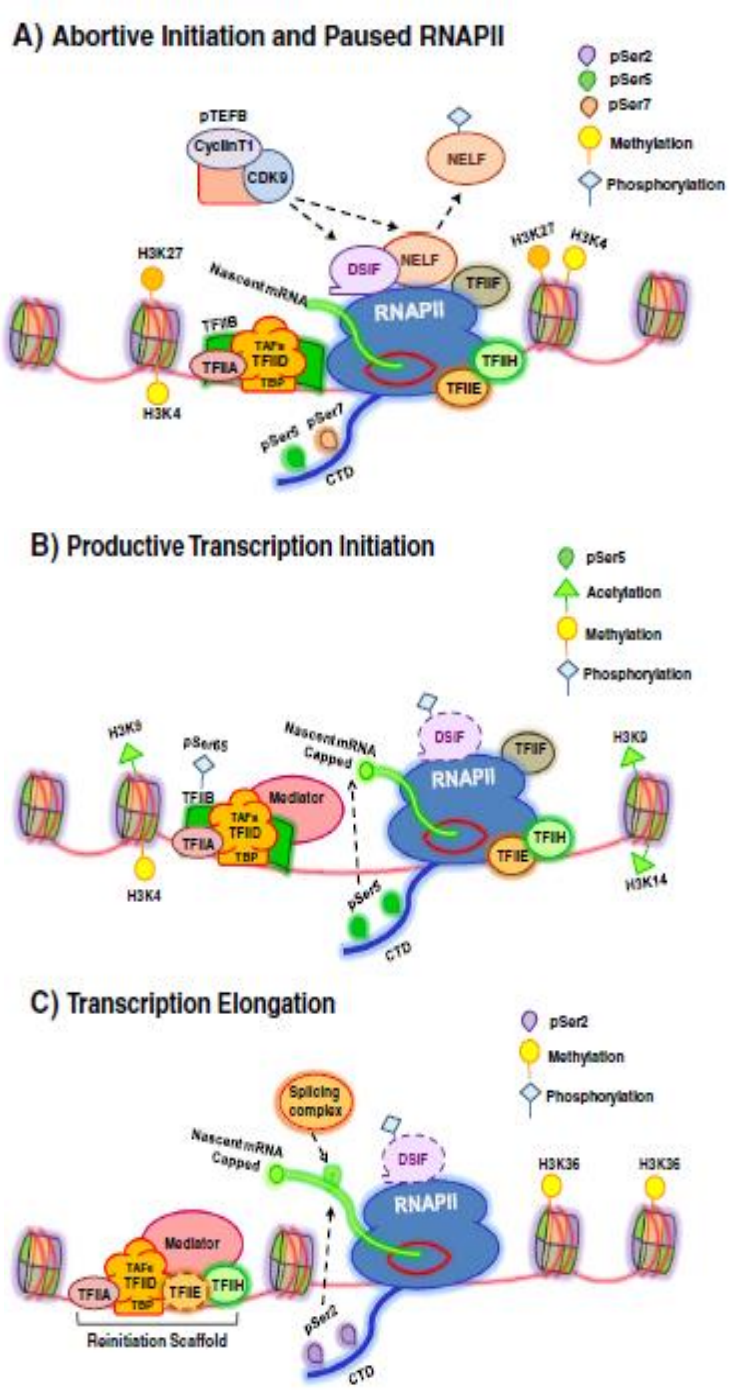
Once transcription starts, the double DNA helix unwinds and RNA pol II reads the template strand (Clancy 2008). During the early elongation phase, there is a checkpoint in which RNA pol II pauses in the promoter-proximal region (30–60 nucleotides downstream of the TSS). Such checkpoints serve as a quality control for the transcript 5′-capping and RNA Pol II modification before productive elongation. Paused RNA Pol II is associated with transcription factors (TFs) that function with negative elongation factor (NELF) and DRB-sensitivity-inducing factor (DSIF) to stabilize the paused Pol II complex. The release of RNA pol II from the pause depends on the positive transcription elongation factor-b (P-TEFb) complex. P-TEFb associates with promoters through interactions with TFs and cofactors, and phosphorylates the carboxy-terminal domain (CTD) of Pol II (Jonkers and Lis 2015). Upon release of RNA pol II, productive elongation begins by adding nucleotides to the 3′ end of the growing strand typically at a rate of 22-25 nucleotides per second. However, these rates can be modulated by different factors such as histones and the number of exons in the gene (Clancy 2008, Jonkers and Lis 2015).

1.4.1.1.4 Mechanism of RNA Pol II termination

The CPF (cleavage and polyadenylation factor) pathway is responsible for the termination of mRNA transcription. This pathway contains a complex of CPF subunits, that interacts with the polymerase (Porrua et al. 2016).

Initially, the RNA strand is cleaved by the CPF complex and this cleavage is coupled with the termination of the transcription that occurs in the consensus sequence AATAAA (Clancy 2008). However, different factors are required to disassemble the elongation complex and the release of RNA Pol II. Processing and termination at the 3’-end are caused by several signals on the nascent RNA, which are recognized by the CPF complex (Figure 14). However, the complete mechanism for termination remains to be fully elucidated. Currently, there are two hypotheses. The first is termed the allosteric model and proposes that upon finding termination signals the polymerase undergoes a conformational change that allows

the enzyme to terminate. The second hypothesis is termed the torpedo model and proposes that the excision of the nascent transcript provides an entry point for a 5'–3' exonuclease (XRN2) that degrades the nascent RNA still bound to the RNA Pol II and causes the termination (Porrua et al. 2016).



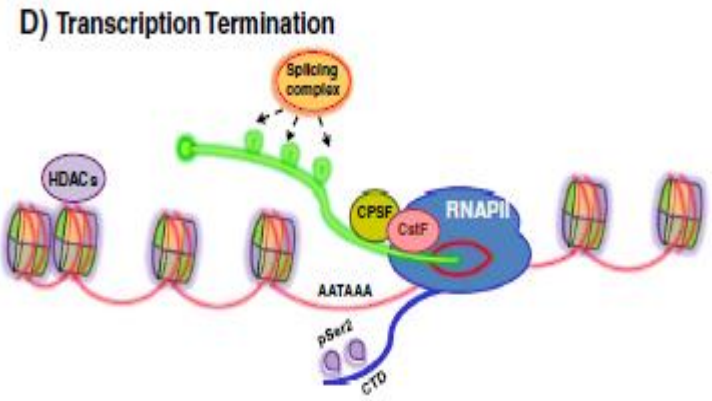


Figure 14. The transcription cycle. (A). Depending on the transcriptional competence of RNAPII, it can potentially enter a paused state. Presence of negative factors such as NELF and DSIF inhibits productive transcription initiation resulting in abortive transcription. Paused RNAPII is also enriched with phospho-Ser7 mark (orange balloon) at its C-terminal domain (CTD). The pTEFb complex alleviates this repression via phosphorylation of NELF and DSIF that results in dissociation of phosphorylated NELF from RNAPII, while DSIF may move along with the elongating RNAPII. (B). RNAPII can switch to a productive initiation mode. Active initiation is dependent on TFIIH-mediated promoter melting (red bubble) and phosphorylation of the CTD repeats (green balloon). Along with the phosphorylation of CTD, productive transcription initiation also requires the phosphorylation of TFIIB. The phospho-Ser5-CTD recruits capping enzyme to the 5′ region of nascent mRNA (green string) that triggers RNAPII-escape from the promoter of the gene. (C). Following promoter clearance, RNAPII proceeds to elongating the transcript while a part of the PIC components remains associated at the promoter forming a preinitiation, GTFs such as TFIIB, TFIIF and likely TFIIE fall off. The elongating RNAPII CTD repeat is progressively phosphorylated. (D). Once the RNAPII reaches a pause signal (poly A) at the gene terminal, 3′ end processing and termination specific complexes such as CPSF and CstF are recruited. The region already transcribed by RNAPII is efficiently reassembled into chromatin with the aid of histone chaperones and deacetylases (HDACs). (From Shandilya and Roberts 2012).

1.5 TFIID

TFIID, a complex of subunits, is a key part of the transcriptional complex and is composed of TATA binding protein (TBP) and a set of 13-14 TBP-associated factors (TAFs) (Green 2000, Cler et al. 2009). TFIID binds to the DNA core promoter, via core promoter elements that can include the TATA box, the initiator (Inr), motif ten element (MTE), downstream

core promoter element (DPE), and the downstream core element motifs (Juven-Gershon and Kadonaga 2010). TFIID can also physically and functionally associate with trans-activating proteins that allow TFIID to play a role as a transcriptional co-activator. These TFIID-activator models propose an induction of PIC assembly (Papai et al. 2011). TAFs, with a molecular weight between 15kDa to 250KDa, were initially named based on their different molecular weights, which differ between different species. In order to standardize the names of TAF protein, Tora and his colleagues proposed a common nomenclature (Table 2) (Thomas and Chiang 2006).

New name	*H. sapiens*	*D. melanogaster*	*S. cerevisiae*	*S. pombe*	*C. elegans*	
					New name	Previous nam
TAF1	$TAF_{II}250$	$TAF_{II}230$	Taf145/130	$TAF_{II}111$	*taf-1*	*taf-1*
TAF2	$TAF_{II}150$	$TAF_{II}150$	Taf150	T38673*	*taf-2*	*taf-2*
TAF3†	$TAF_{II}140$	$TAF_{II}155$	Taf47		*taf-3*	C11G6.1*
TAF4†	$TAF_{II}130/135$	$TAF_{II}110$	Taf48	T50183*	*taf-4*	*taf-5*
TAF4b†	$TAF_{II}105$					
TAF5	$TAF_{II}100$	$TAF_{II}80$	Taf90	$TAF_{II}72$	*taf-5*	*taf-4*
TAF5b				$TAF_{II}73$		
TAF5L	PAF65α	Cannonball				
TAF6†	$TAF_{II}80$	$TAF_{II}60$	Taf60	CAA20756*	*taf-6.1*	*taf-3.1*
TAF6L	PAF65α	AAF52013*			*taf-6.2*	*taf-3.2*
TAF7	$TAF_{II}55$	AAF54162*	Taf67	$TAF_{II}62$/PTR6	*taf-7.1*	*taf-8.1*
TAF7L	TAF2Q				*taf-7.2*	*taf-8.2*
TAF8†	BAB71460*	Prodos	Taf65	T40895*	*taf-8*	ZK1320.12*
TAF9†	$TAF_{II}32/31$	$TAF_{II}40$	Taf17	S62536*	*taf-9*	*taf-10*
TAF9L	$TAF_{II}31L$					
TAF10†	$TAF_{II}30$	$TAF_{II}24$	Taf25	T39928*	*taf-10*	*taf-11*
TAF10b		$TAF_{II}16$				
TAF11†	$TAF_{II}28$	$TAF_{II}30\alpha$	Taf40	CAA93543*	*taf-11.1*	*taf-7.1*
TAF11L					*taf-11.2*	*taf-7.2*
TAF12†	$TAF_{II}20/15$	$TAF_{II}30\alpha$	Taf61/68	T37702*	*taf-12*	*taf-9*
TAF13†	$TAF_{II}18$	AAF53875*	Taf19	CAA19300*	*taf-13*	*taf-6*
TAF14			Taf30/ANC1#			
TAF15	$TAF_{II}68$#					
BTAF1	$TAF_{II}170$	Hel89B	Mot1	T40642*	*btaf-1*	F15D4.1*

The TAF nomenclature for *C. elegans* is indicated on the right two columns, shaded in grey. (Adapted from Tora, 2002).
†TAFs with histone fold domains.

Table 2. Nomenclature of TAFs involved in RNA polymerase II-mediated transcription. (From Thomas and Chiang 2006).

TAFs can also regulate transcription through recognition and binding to the core promoter (Malkowska et al. 2013). Associations among TAF-Inr, TAF-DPE and TAF-DCE (Downstream Core Element) can confer the ability of TFIID to recognize TATA-less promoters (Thomas and Chiang 2006). Many TAFs also have a histone fold domain that contributes to the recognition of core promoter elements. In order to maintain the integrity of the TFIID complex and to induce TAFs dimerization, the histone fold domain mediates many subunit interactions (TAF4-TAF12, TAF6-TAF9, TAF10-TAF3, TAF10-TAF8, and TAF11-TAF13) (Birck et al. 1998, Lavigne et al. 1999, Gangloff et al. 2000, Gangloff et al. 2001, Gangloff et al. 2001, Gangloff et al. 2001, Thuault et al. 2002, Werten et al. 2002).

Moreover, crystallographic and *in vitro* studies have been shown a histone octamer-like TAF complex existing within TFIID that includes TAF4-TAF12 and TAF6-TAF9 (Hoffmann et al. 1996, Selleck et al. 2001).

1.5.1 TAF6

TAF6 is a transcription factor encodes by a gene containing 12300 base pairs, located on human chromosome 7. TAF6 is a subunit of the TFIID complex in the general transcription of RNA pol ll having two major domains, a HEAT repeat domain and a histone fold domain that can be divided into two sub-domains: a small middle domain and a larger domain. Mutations analyses demonstrate that the larger histone fold domain modulates the heterodimeric interaction between TAF6/TAF9 which in addition of TAF5 enhances the modulating effect of TAF6 (Scheer et al. 2012). It has also been reported that this dimeric complex (TAF6/TAF9) shows DPE-binding specificity in electrophoretic mobility shift assays (Figure 15) (Shao et al. 2005).

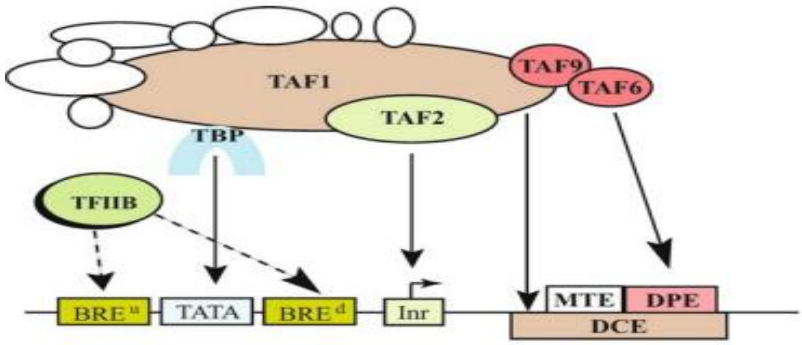


Figure 15. Recognition of core promoter elements by TFIID and TFIIB. The interactions between TFIID and TFIIB with the core promoter elements. (From Thomas and Chiang 2006).

One study revealed the crystal structure of TAF6 and showed that the HEAT repeat domain of TAF6 forms a homodimer that bridges TAFs (TAF1, TAF2 and TAF7) that interact with the downstream promoter (Louder et al. 2016). However, in that report they were not able to detect the density of the histone-fold domain of TAF6 that serves to interact with TAF9. Taking all together, available data suggest that the histone fold of TAF6 is flexible but not crucial for the structural integrity of the TFIID core (Louder et al. 2016). This coincides with

the fact that the human TAF6δ isoform, lacking a critical part of its histone fold domain, integrates into an active TFIID complex containing all TAFs except TAF9 (Figure 16) (Bell et al. 2001).

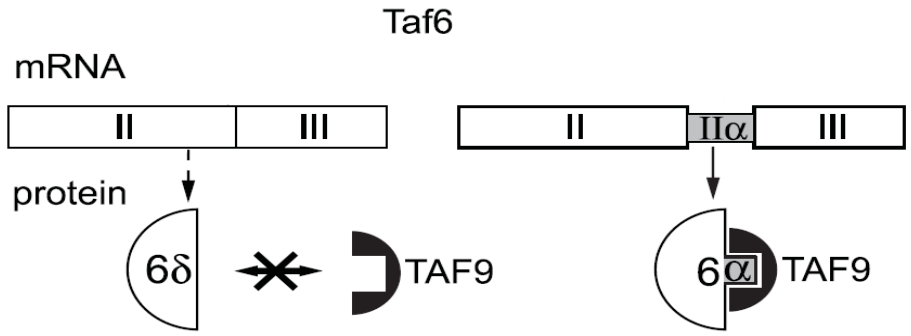


Figure 16. TAF6 alternative splicing modified interaction with his dimer partner TAF9. The protein produced by the major splice variant, TAF6α, can interact with the TFIID subunit, TAF9 via its histone fold domain. In contrast, TAF6δ lacks 10 amino acids of helix 2 of its histone fold motif and therefore cannot interact with TAF9. (From Wilhelm, Pellay et al. 2008).

Furthermore, TAF6 has been reported as essential for viability in yeast, Arabidopsis and *Drosophila*, as studies have shown that deletion of the TAF6 gene is lethal (Michel et al. 1998, Aoyagi and Wassarman 2001, Lago et al. 2005). Our laboratory has recently also shown that TAF6 is essential for viability in human cells (Kamtchueng et al. 2014). Another feature of TAF6 is that it produces five isoforms as a result of alternative splicing (Figure 17). The first three isoforms (TAF6α, TAF6β and TAF6γ) were detected by DNA sequence analysis (Weinzierl et al. 1993). The most abundant splicing variant is TAF6α, which encodes a 677 amino acid protein. TAF6β isoform encoding a 726 amino acid protein and TAF6γ isoform encoding a 667 amino acid protein. TAF6α, TAF6β and TAF6γ contain the first constitutive exon 2 and the alternative exon 2. TAF6β, unlike the TAF6α isoform, contains an upstream start codon that introduces 49 amino acids in addition to the NH_2-terminal of the protein. However, TAF6γ exhibits 10 amino acids less than TAF6α due to the loss of the alternative part of exon 13. In addition, the other splicing variant that has been reported is the TAF6ε isoform encoding a 716 amino acid protein and the TAF6δ isoform that encodes a 667 amino acid protein. Both isoforms (TAF6ε and TAF6δ) lack 10 amino acids after the alternative splicing that eliminate part of the exon 2 alternative. The difference between

TAF6ε and TAF6δ is that TAF6ε contains the same 49 additional amino acids as TAF6β. In the case of TAF6δ, the deletion of 10 amino acids disrupted the second helix of the histone fold domain. Therefore, it cannot interact with its TAF9 dimer partner, but can continue to interact with TFIID and other TAFs such as TAF1, TAF5 and also TBP (Weinzierl et al. 1993, Bell et al. 2001, Wang et al. 2004).

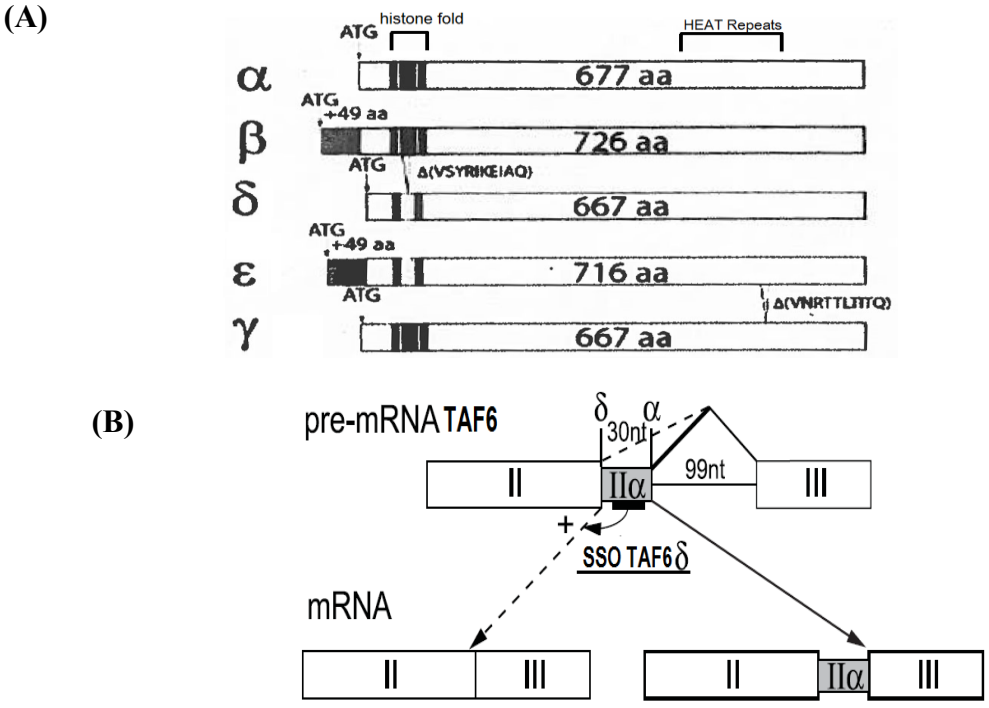


Figure 17. Isoforms of TAF6. (A) Schematic representation of the five TAF6 isoforms. Representation of the histone fold motif and the HEAT repeats (c-terminal domain) are indicated by clasps. Narrow rectangles represent cDNA, thick rectangles indicate protein sequences and start codons (ATG) are indicated with arrows. (B) The region of the TAF6 pre-mRNA that includes two alternative 5' splice sites (SSs) that produce either, the constitutive α splice variant or the alternative δ splice variant is schematically depicted. The selection of an intron-proximal α 5' splice site (SS) results in production of the isoform of the major TAF6 isoform (at right), whereas the selection of the proximal δ 5' SS results in the production of the minor δ isoform (at left). The SSOs that base pair with the alternative exon forces splicing from the distal 5' SS and induces the expression of the endogenous TAF6d isoform (at left). (Adapted from Wang, Nahta et al. 2004, Wilhelm, Pellay et al. 2008).

1.5.2 TAF6δ

It has been reported that the TAF6δ isoform is specifically expressed in apoptotic conditions (Bell et al. 2001). The fact that TAF6δ is generated from TAF6 pre-mRNA alternative splicing has been demonstrated by using an experimental system termed SSOs (splice-switching oligonucleotides that act by hybridizing to pre-mRNA sequences and blocking access to transcript by splicing factors) (Kole et al. 2004, Bauman et al. 2009). SSOs can be used to force the expression of the endogenous TAF6δ isoform (Figure 17B). The SSOs oligonucleotides bind the alternative exon 2 and interfere with the normal functioning of the spliceosome, which means that the oligonucleotide induces the skip of the alternative exon 2, thus resulting in a short RNA (δ isoform). Contrast microscopy showed that the expression of TAF6δ resulted in loss of cell adhesion and cells with apoptotic characteristics, such as membrane blebbing. It was further demonstrated that TAF6δ increased cell death in several cancer cell lines (HeLa, Saos-2, H1299, HL-60, A549 and HCT-116) (Wilhelm et al. 2008). In addition, SSOs RNA technology showed that TAF6δ can control apoptosis independently of the tumor suppressor p53 (Wilhelm et al. 2008). Transcriptome analysis performed in our laboratory showed that endogenous TAF6δ defined a pro-apoptotic transcriptome signature (Wilhelm et al. 2010). The TAF6δ-driven transcriptome landscape showed statistically significant enrichment of genes acting in several different pathways, including the Notch pathway, oxidative stress, integrin's, p53, apoptosis and angiogenesis (Figure 18).

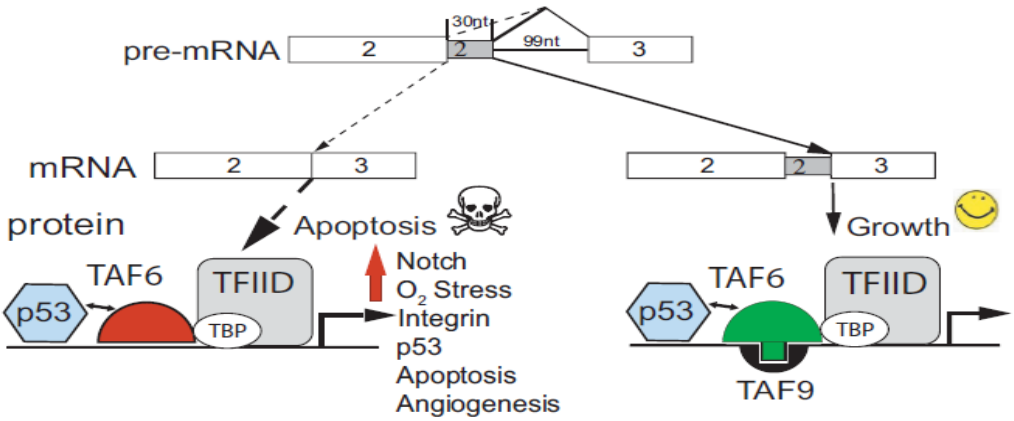


Figure 18. A model for the TAF6δ pathway. A hypothetical model coupling changes in gene expression to cell death via the TAF6δ pathway of apoptosis. (From Wilhelm, Kornete et al. 2010).

Our laboratory has recently shown that the TAF6δ-driven transcriptome signature is distinct from that observed when the major TAF6 isoform, TAF6α was depleted by siRNA (Kamtchueng et al. 2014). These results underscore the importance of TAF6δ induction as opposed to the simple loss of total TAF6 protein in the control of a physiological cell death transcriptional program. While TAF6δ's role is the induction of apoptosis is well established, the physiological role of TAF6δ in living organisms remains obscure. Since the genes of the Notch pathway were the most statistically significant enriched in response to TAF6δ expression, we hypothesized that TAF6δ may influence and possibly activate the Notch pathway. Another clue suggesting the activation of the Notch pathway by TAF6δ was the observation that the classical Notch target gene, Hes1 is induced by TAF6δ (Wilhelm et al. 2010). However, the specific implication of the Notch signalling pathway and the molecular mechanism for apoptosis induction by TAF6δ are presently unknown. Consequently, in this study, we asked whether TAF6δ induction can activate Notch signalling and whether Notch signalling contributes to TAF6δ-dependent apoptosis. The results of my master's work extend our laboratory's earlier work showing that genes from the Notch signalling pathway are overrepresented in the TAF6δ-driven transcriptome signature in HeLa cells (Wilhelm, 2010), by revealing that the regulation of Notch-related genes occurs in cancer cell lines of multiple tissue origins. Most importantly, my results show that Notch2 is activated by TAF6δ, and that Notch signalling can contribute to TAF6δ-induced apoptosis.

2 HYPOTHESIS

Apoptosis plays an important role in the formation of organs and tissues (Rajesh P. Rastogi 2009). Additionally, several signalling pathways can activate the apoptotic process, including the Notch pathway (Zweidler-McKay et al. 2005, Robert-Moreno et al. 2007). However, Notch signalling implication in tumorigenesis is not well defined yet. Depending of the context, Notch pathway has been demonstrated to have oncogenic or tumor suppressor effect (Previs et al. 2015). This signalling cascade shows a tumor suppressor effect in cervical cancer (Yao et al. 2007). Indeed, it was discovered that Notch expression is reduced in cervical cancer cells, suggesting that specific down-modulation of Notch pathway is required in the tumorigenesis process (Talora et al. 2002, Sakamoto et al. 2012). In addition, in cervical cancer cells, it has been reported that the activation of the Notch pathway results in the inhibition of tumor growth through induction of apoptosis and cell cycle arrest (Yao et al. 2007). In particular, in HeLa cells, it has been shown that activation of the Notch pathway decreases cell proliferation and induces apoptosis (Wang et al. 2007).

TFIID, a macromolecular complex, is a fundamental part of the transcriptional complex and it is composed of TATA binding protein (TBP) and a set of 13-14 TBP-associated factors (TAFs) (Cler et al. 2009). TAFs can regulate transcription through recognition and binding to the core promoter (Malkowska et al. 2013), and it has been reported that the TAF6δ isoform generated by the alternative splicing of TAF6 pre-mRNA is expressed only under apoptotic conditions (Bell et al. 2001). Furthermore, it was demonstrated that TAF6δ induces cell death in several cancer cell lines (HeLa, Saos-2, H1299, HL-60, A549 and HCT-116), independently of the p53 tumor suppressor (Wilhelm et al. 2008). Importantly, the endogenous expression of TAF6δ on the global transcriptome landscape reveals a statistical enrichment in several genes that act in different pathways including the Notch pathway (Wilhelm et al. 2010). In contrast, with a transcriptome reporting the depletion of the major TAF6 isoform, TAF6α does not result in significant changes in genes in the Notch pathway (Kamtchueng et al. 2014). In our previous microarray data, we observed increases in genes of the Notch pathway, including the most studied Notch target gene, Hes1. However, the whether or not the Notch signalling pathway is activated by TAF6δ, and whether Notch

signalling contributes to the induction of apoptosis by TAF6δ are presently unanswered questions. Therefore, my project was to test the hypotheses that TAF6δ activates Notch signalling and whether the Notch pathway participates in the induction of apoptosis.

In order to verify this hypothesis, we have two general objectives:

A) General Objective

Does TAF6δ expression cause activation of the Notch pathway?

B) General Objective

Can Notch activation contribute to TAF6δ-dependent apoptosis?

3 MATERIAL AND METHODS

3.1 Cell culture

HeLa cells were grown in DMEM (Dulbecco's Modified Eagle Medium) containing 2.5% CS (calf serum) and 2.5% FBS (foetal Bovine serum). Hs-578-T and MDA-MD-231 cell lines were cultured in DMEM with 1% Glutamine and 10% and 15% of FBS, respectively. Panc1 cells were grown in DMEM supplemented with 10% FBS, 1% Glutamine, 1% Sodium Pyruvate and 1% Hepes (table 3).

Table 3. Cancer cell lines used in this study

Cancer cell line	Origin	Provider
HeLa	Cervix Adenocarcinoma	Thanks to the IGBMC institute (Strasbourg)
Hs-578-T	Breast Carcinoma	Thanks to Pr. Benoît Chabot
MDA-MB-231	Breast Adenocarcinoma	Thanks to Pr. Benoît Chabot
Panc1	Pancreas Epithelioid Carcinoma	Thanks to Pre. Marie-Josée Boucher

3.1.1 Transfections with SSOs (Splice Switching Oligonucleotides)

2'-O-methyl-oligoribonucleoside phosphorothioate antisense 20-mers were purchased from Sigma-Proligo, USA. "SSOs control" and "SSOs TAF6δ, also called SSOs T6-1" have been described previously (Wilhelm et al. 2008). "SSOs T6-3" 5'-CUGUGCGAUCUCUUUGAUGC-3' targets the 3' part of the alternative exon 2 of TAF6 and induces TAF6δ production. "Control SSOs" 5'-AUGGCCUCGACGUGCGCGCU-3' and "Control SSOs-2" 5′ ACGGUCCGUUAGCGUGCCGC 3′ are a scrambled oligo's used as a negative control.

The cells were seeded at densities of 70,000 cells/0.5ml in 24-well plates and cultured overnight at 37ºC with 5% of CO_2. Twenty-four hours later the medium was replaced by 350µl of Opti-MEM (Minimal Essential Medium) (Invitrogen) and put it back at the incubator at 37ºC with 5% of CO_2 whereas the mixes where prepared. The SSOs were transfected with lipofectamine 2000 (Invitrogen). First 0.8µl of lipofectamine was mixed with 50µl of Opti-MEM for 5 minutes at room temperature while a second mix was prepared contain SSOs plus 50µl of Opti-MEM at a final concentration of 100 nM. Next, the lipofectamine mix were combining with SSOs mix for 20minutes at room temperature. Subsequently, 100µl of the complex (liposomes-SSOs) were added drop by drop to each well delicately. Eighteen to twenty-four hours after the cells were harvested for analysis. All transfections were performed in Opti-MEM.

3.2 Blocking Notch pathway by GSI treatment

DAPT (N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenyl glycine t-butyl ester) and S2188, γ-secretase inhibitors were purchased from Sigma, USA. They were used to block Notch signalling transduction mediated through γ-secretase cleavage of Notch receptor. Cells in logarithmic growth were seeded at densities of 70,000 cells/0.5ml in 24-well plates and cultured in the presence of the GSI (50µM DAPT or 15µM S2188) and control cells were treated with DMSO (Dimethyl sulfoxide) for 1h, before the SSOs were transfected. Eighteen to twenty-four hours later, the cells were harvest to analyze the expression of Notch ligands, receptors and target genes (mRNA levels) and Notch1-2, Hes1 (protein level) and apoptosis induction.

3.2.1 Activation of Notch Pathway through EGTA treatment

EGTA (ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetra acetic acid: 5mM) a calcium-chelator from Sigma Aldrich. It was used for 15 minutes to activate the cleavage of the Notch receptors then the medium was replaced by fresh culture media (DMEM) for 1h before harvest the cells.

3.3 Antibodies

Mouse monoclonal antibody against TAF6δ (37TA1C2; 1:1200) have been described (Bell et al. 2001). Rabbit polyclonal antibody against Hes1 (AB5702; 1:2000) and mouse monoclonal antibody against TATA Box-binding protein; TBP (clone 5FT1-1C2; 1:2500) were purchased from Millipore. Rabbit monoclonal antibody against Cleaved Notch1 (Val1744 (D3B8) #4147; 1:1000) and rabbit monoclonal antibody against Notch2 (D67C8 #4530; 1:1000; it recognizes both the full-length and the transmembrane/intracellular region) were purchased from Cell Signaling Technology. Rabbit polyclonal antibody against Notch2 (8926; 1:1000; the epitope is only exposed after gamma secretase cleavage) were purchased from Abcam. Secondary antibodies used in western blot: anti-mouse and anti-rabbit IgG-horseradish peroxidase (HRP) were purchased from Jackson Immunoresearch Laboratories. Secondary antibodies used in immunofluorescence: Alexa Fluor 546 goat anti-mouse IgG (1:1200) and Alexa Fluor 488 goat anti-rabbit IgG (1:400) were purchased from Molecular Probes.

3.4 Extraction of RNA

The cells previously transfected were collected for RNA extraction. The Opti-MEM medium that contained the floating cells was placed in an Eppendorf tube. Two hundred and fifty microliters of Trizol (Invitrogen) were added to the wells in order to retrieve all the adherent cells for 5min at room temperature after which the Eppendorf with medium were centrifuged for 30 seconds at 10,000 rpm. The supernatant was discarded; the mix adherent cells-Trizol was added to the pellet. Subsequently, 50µl of chloroform were added, mixed vigorously and let it for 2 minutes on ice. Samples were then centrifuged for 15minutes at 13,000 rpm to separate the phases. The aqueous phase that contained the RNA was transferred onto 125µl of isopropanol, mixed and centrifuge for 15minutes at 13.000rpm to precipitate the RNA. The pellet obtained was washed with 1 ml of ethanol 75% and centrifuge for 5 minutes at 8.000rpm. The pellet was then dried for 10 minutes at room temperature and resuspended with 12µl of nano water.

3.4.1 RT-PCR (Reverse transcription polymerase chain reaction)

Total RNA was extracted using Trizol (Invitrogen) as previously described. 1µg of total RNA was reverse transcribed using MMuLV reverse transcriptase (from Catherine Desrosiers). 1µg of total RNA was mixed to hybridize with 2µl of Oligo dT (20µM) at a final volume of 12µl for 2 minutes at 95ºC. Then, 8µl of master mix (4µl of 5x MMuLV Buffer, 2µl of DNTP´s 10mM, 1µl of DTT (dithiothreitol) 5µM and 0.6µl of MMuLV reverse transcriptase) were added and complemented with nano water until final volume of 20µl for 1h at 42ºC.

3.4.2 PCR (Polymerase chain reaction)

The complementary DNA (cDNA) previously obtained was used as a template on the PCR. 3µl of cDNA were mixed with 22µl of solution buffer that contained: 2.5µl of 10x PCR buffer, 0.5µl of DNTP´s 10mM, 0.25µl Taq DNA Polymerase (from Catherine Desrosiers) and 0.5µl of each oligo 20µM (T6-1B: 5´- ATGGGCATCGCCCAGATTCAGG -3´ and T6-2E: 5´-AAGGCGTAGTCAATGTCACTGG-3´) that amplified TAF6α and TAF6δ. The reaction PCR conditions were: 95ºC, 3 min; denaturation; 25 cycles of 94ºC for 1 min; denaturation, 58ºC for 45 sec; hybridization, 68ºC for 50 sec; final extension at 68ºC for 5 min. Primers used for amplification of both TAF6α and TAF6δ have been described previously (Wilhelm et al. 2008). The PCR products were qualitatively analyzed through an electrophoresis on polyacrylamide gel 15% to confirm TAF6δ induction after SSOs transfection in each experiment. The polyacrylamide gel was performing as a mix: 3ml of acrylamide 30%: bis acrylamide (30: 0.8), 1.2ml of TBE 5X (Tris base 0.09M, EDTA 0.5M and boric acid 0.089M), 42µl of Ammonium persulfate (APS), 2.2µl of TEMED and complemented with nano water until final volume of 6ml. 10µl of the PCR product was loading on the gel, migrated and stained with ethidium bromide (EtBr). The images were recorded using a UV photo-doc system.

3.4.3 qPCR (Real time PCR)

Real time PCR was performed on cDNA previously obtained by RT-PCR. Real-time PCR was made in a final 20µl reaction containing 10 ng of cDNA with 10 µl of the 2X master mix buffer (50ml Stock Buffer: 600µl Tris pH 8.0 1M, 1 ml KCL 2.5M, 400µl MgCl2 1M, 2.84g Trealose (TRE222, Bioshop), 100µl Tween 20, 500µl bovine serum albumin (BSA) 20mg/ml (NEB #B9001S), 1µl SYBR® Green (Life Technologies # S7563)), 0.4µl of DNTP´s 10mM, 0.6µl of each primer 5 µM, 0.2µl Klentaq (from Catherine Desrosiers) and 4.2µl of nano water, mixed in 96 well plates (PCR® microplate, 96 well Flat Top, clear (Axygen, INC, USA)). Real-time PCR relative quantification assay was running for 2 minutes at 95ºC, followed by 40 cycles of 15 seconds at 95ºC, 15seconds at 58ºC, and 20 seconds at 68ºC after 15 seconds at 95ºC, 15 seconds at 60ºC, and 15 seconds at 95ºC. Relative quantity of target genes was calculated using the comparative C_T ($\Delta\Delta C_T$) method using hRPLPO (large ribosomal protein) as the internal control. Primer sequences are listed in Table 4. The PCR products were run on a 2% agarose gel to confirm the size of the product.

Table 4. Primer sequences, annealing temperatures and amplicon sizes for qPCR analysis used in this study

Primer	Sequence (5´-3´)	Annealing temperature (°C)	Amplicon size (pb)
Dll1	ATGTGATGAGCAGCATGGATT	58	197
	GGTGTGTGCAGTAGTTCAGGTC		
Dll3	CCCTTCCTCGATTCTGTCCG	60	160
	ACCTCCTCAAGCCCATAGGT		
Dll4	GCC AGA GGC CTT GCC ACC AG	63	183
	CGC TTC TTG CAC AGG CGG GA		
Jagged1	AAC GAC CGC AAC CGC ATC GT	63	170
	TTC AGC GTC TGC CAC TGC CG		
Jagged2	CGG CGT CAA CTG GTT CCG CT	63	288
	CCG TGT GGG AAC GGA GTG CC		
Notch1	GCG GTC CCA ACT GCC AGA CC	63	284
	GCA CGG GCT CAG AAC GCA CT		
Notch2	CCCACAAAGCCTAGCACCAA	60	177
	ATTGGAAGGCACCTTGTCCC		
Notch3	AGC GTT GTC AGC GGT GGA GC	63	488
	CGT CGC CCT GTG GTG GTG TC		
Notch4	ACTTGGTCCGTAGACTTGGC	58	521

	TCTGCTCTGGTGGGCATACAT		
Hes1	TGCTACCCCAGCCAGTGTCAA	58	150
	AGAGCATCCAAAATCAGTGTTTTCAGC		
Hey1	CTGAGCAAAGCGTTGACAAA	60	212
	TCCACCAACACTCCAAATGA		
Hey2	AGGCTACTTTGACGCACACG	58	153
	CAAGTGCTGAGATGAGACACAAG		
Bim	ATGTCTGACTCTGACTCTCG	58	173
	CCTTGTGGCTCTGTCTGTAG		
Noxa	TCCTGAGCAGAAGAGTTTGG	58	163
	GGAGATGCCTGGGAAGAAGG		
Acrc	CTCATGGTGACGCATGGAAG	58	144
	AGCAGCCAATCCTCGTTTTG		
hRPLPO	GCAATGTTGCCAGTGTCTG	58	142
	GCCTTGACCTTTTCAGCAA		

3.5 Western Blot

HeLa Cells previously transfected were collected for protein extraction. Floating cells were transferred in an Eppendorf. The cells were then washed with 500µl of PBS 1X (NaCl 137mM, KCl 2.7mM, Na_2HOP_4 4.3mM and KH_2PO_4 1.47mM pH 7.4) and centrifuged twice for 1 minute at 4000rpm while the monolayer cells were lysed in 20µl of white laemmli sample buffer 1.5X (150mM Tris, 15% glycerol and 3.75% SDS) after the pellet obtained by centrifugation was mix with the lysates-cells and boiled for 5 minutes at 95ºC and sonicated (under amplitude of 90 for 2.5 minutes with 10 seconds break every 20 seconds). Subsequently, protein concentrations were determined with BCA Protein Assay Kit (Thermofisher, scientific; #23221). Five to twenty micrograms of protein lysates were electrophoresed with migration buffer (Tris-base 0.025M, glycine 0.192M and SDS 0.1%) on 7.5% and 12% SDS-PAGE (Resolving buffer: Tris 1.5 M pH8.8, SDS 0.4% and acrylamide/bisacrylamide (30:0.8) and Stacking buffer: Tris 0.1M pH 6.8, SDS 0.4% and acrylamide/bisacrylamide (30:0.8)) at 100 volts during 10 minutes and after 150Volts for 40 minutes, before transferred to Nitrocellulose membrane (PROTRAN, PerkinElmer) with transfer buffer (Tris-base 0.01M, glycine 0.077M and ethanol 20%) during 1h at 115Volt. PBS-Tween 0.05% containing 5%nonfat milk was used to block nonspecific binding for 1h at room temperature. Membranes were incubated overnight at 4ºC with previously described

primary antibodies against Hes1, Cleaved Notch1, Notch2 or TBP. Next day, the membranes were washed 3 times with PBS-Tween 0.05% for 5 minutes and a second incubation was conducted with HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) for 2h at room temperature. The bound antibodies were visualized using an enhanced chemiluminescence kit (Western Lightning Plus-ECL; PerkinElmer).

3.6 Immunofluorescence

HeLa cells (60.000cells/0.5ml) were grown on cover slips pre-treated with 200µg/ml of L-polyLysine (Sigma #P6282) for 24h and then washed twice with phosphate-buffered saline (PBS) before the SSOs were transfected. 18h after cells were washed twice with PBS and subsequently fixed in 2% of paraformaldehyde (PFA) for 6min, permeabilized with PBS-0.1% Triton X-100 (PBS-Tx twice for 10 min and then incubated for 30 min with blocking buffer (PBS-Tx containing 1% bovine serum albumin (BSA) and 0.5% fish gelatin (Sigma-Aldrich)). Cells were then incubated one hour at room temperature, with each of the following antibodies diluted in blocking buffer; anti-TAF6δ (37TA1C2: 1:1200), Alexa Fluor 546 goat anti-mouse IgG (1:1200) secondary antibody (Molecular Probes), anti-Notch2 (Ab8926: 1.1000), Alexa Fluor 488 goat anti-rabbit IgG (1:400) secondary antibody (Molecular Probes), followed by three washes with permeabilization buffer between each antibody. Cells were then treated with Hoechst 33342 (2 µg/ml) and visualized by fluorescence microscopy (Leica DM2500 Optigrid). Total nuclear fluorescence analyses were performed by CellProfiler 2.1.1 software, http://cellprofiler.org/previous_releases/.

3.7 Apoptosis assays

HeLa cells were seeded at densities of 70,000 cells/0.5ml in 24-well plates and cultured overnight at 37ºC. After being treated with GSI (DAPT) and SSOs were transfected as described above. Eight-teen hours after, floating and trypsinized cells were collected, washed with cold PBS and centrifuged at 8000 rpm for 1 min at room temperature. The cell pellet was fixed using 3% formaldehyde, incubated 10 min at 37ºC and centrifuged twice at 8000 rpm for 1 min at room temperature. Then, the pellet was permeabilized with 300µl of cold

Methanol 100% for 30 min on ice and subsequently blocking buffer that contained (PBS1X; BSA 0.5%) was added. The cells were next centrifuged twice before resuspended in 100µl of blocking buffer that contained cleaved caspase-3 antibody diluted (1:2000) (Cell Signaling Technology; #9661) and incubated overnight at 37ºC. 1 ml of blocking buffer was then added to the cells and centrifuged. The pellet was resuspended in 100µl of blocking buffer that contained secondary antibody (α-Rabbit- Phycoerythrine; Jackson Immunoresearch Laboratories) diluted (1:100) and incubated for 60min at room temperature in the dark. Subsequently, 1 ml of blocking buffer were added to the cells and centrifuged. Then stained cells were resuspended in 100µl of PBS and analysis were performed using Flow Cytometry (Becton Dickinson FACScan) following the manufacturer's recommendations.

3.8 Microarray Analysis of Gene Expression

Transcriptome analysis was performed as we previously detailed (Wilhelm et al. 2008), using the NeONORM normalization method (Noth et al. 2006). Free parameter *k* was set to 0.2. The AB1700 data generated for this study on an Applied Biosystems Microarray platform were annotated according to the published procedure (Noth and Benecke 2005). For comparative cellular process inference analyses, the combined TAF6δ pathway activation and inhibition of the Notch pathway were performed with Gene Ontology (GO) and KEGG annotations as we previously detailed (Wilhelm et al. 2010). P-values were determined using a hypergeometric distribution and a null hypothesis of a random set of genes with identical size. Microarray data for the gene sets analyzed herein are provided in Additional Files. The transcriptome-wide microarray data for all of the experiments described here were deposited in the database http://mace.ihes.fr under accession numbers: Notch signature: **2547351260**; TAF6δ signature: **2937831950**.

3.9 Statistical analyses

The Student´s t-test for estimation of statistical significance for all data except the microarray analysis. P-values within ($P < 0.05$) are marked by one asterisk. Whole genome gene expression data were analyzed using the CDS test (CDS: a fold-change based statistical test

for concomitant identification of distinctness and similarity in gene expression analysis (Tchitchek et al. 2012), dimensionality reduction was performed using SVD-MDS (Becavin et al. 2011).

4 RESULTS

A) General Objective

Does TAF6δ expression cause activation of the Notch pathway?

4.1 Objective 1: To determine the impact of Notch pathway inhibition on the TAF6δ-driven transcriptome changes in HeLa cells.

4.1.1 Notch signalling impacts TAF6δ-driven transcriptome changes

Given that TAF6δ regulates the expression of genes in the Notch pathway, including classical direct Notch target genes such as Hes1 (Wilhelm et al. 2010), we sought to determine whether Notch signalling is activated by TAF6δ expression. If the Notch pathway is activated by TAF6δ, Notch activity could potentially contribute to the transcriptome changes induced by TAF6δ. To directly test the impact of Notch signalling on TAF6δ-driven transcriptome changes we employed microarray technology to measure transcriptome-wide gene expression, together with SSOs (Splice-Switching oligonucleotides) to induce the expression of the endogenous TAF6δ splice variant as previously reported (Wilhelm et al. 2008). Notch signalling was inhibited using the GSI (γ-secretase inhibitor) in HeLa cervical carcinoma cells. We measured the impact of GSI (S1288) treatment on control cells (treated with a scrambled SSOs) and on cells where SSOs were used to induce TAF6δ. GSI treatment of control cells caused both increases and decreases in gene expression with the majority of statistically significantly changed transcripts displaying increased expression (Figure 19A, left heat map red versus blue). In contrast, GSI treatment of cells where endogenous TAF6δ was induced showed a reduction in expression of the majority of regulated transcripts (Figure 19A, right heat map red versus blue), suggesting that TAF6δ-induced gene transcription is selectively reduced when Notch signalling is inhibited. Further analysis of the transcriptome changes showed that 491 genes were statistically significantly regulated by TAF6δ and, of these, only 116 remained significantly changed in the presence of GSI (Figure 19B). Globally, the majority of the 491 TAF6δ-regulated genes had a reduced change in expression (Figure 19C, shown in red). Despite the fact that γ-secretase inhibition resulted in the reduced induction of numerous TAF6δ-regulated genes, the induction of a subset of TAF6δ-

dependent genes was augmented in the presence of GSI (Figure 19 C, shown in green), ruling out the possibility that GSI could cause a non-selective reduction in transcription.

We performed gene ontology analysis on the 375 genes that were TAF6δ-regulated and whose change in expression was reduced by GSI treatment to shed further light on the function of genes in that subset. The ontology analysis revealed a statistically significant enrichment of genes in the ontology-classes of chromatin packaging, cell proliferation, and nucleoside/nucleotide metabolism (Figure 19D).

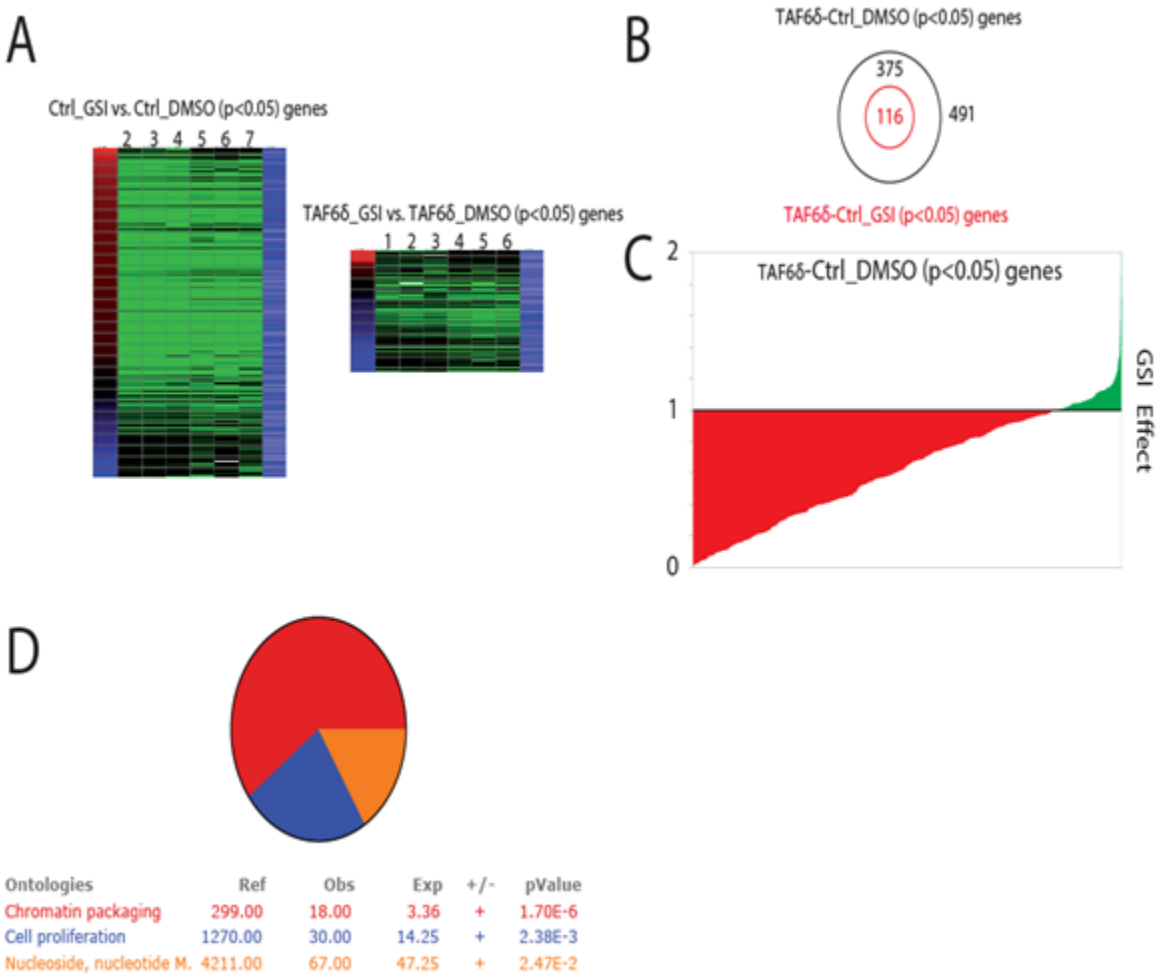


Figure. 19. A fraction of TAF6δ-regulated transcription depends on Notch signalling. (A) Heat map of statistically significantly (P <0.05) regulated genes in response to the GSI (S2188) (lanes 2 – 4) versus DMSO treatments (lanes 5 – 7) during TAF6δ induction by SSOs TAF6δ (right) or in control DMSO treated cells (left). The darkness of lane 8 reflects decreasing P-values. (B) A Venn diagram indicates a subset of TAF6δ-regulated genes (491). The black circle indicates 375 genes regulated by TAF6δ in the absence of GSI while the inner red circle indicates the subset of 116 genes whose regulation remains statistically

significant (P <0.05) in the presence of GSI. (C) Global impact of GSI on TAF6δ-regulated transcription. Fold change (Y-axis) was normalized with microarray data obtained from GSI-free experiments. A value of 1 represents no impact of the inhibitor on gene expression. The x-axis represents the 491 TAF6δ-regulated ordered by the magnitude of the effect of the GSI from negative (red) to positive (green). (D) Gene ontology analysis of the 375 genes whose regulation by TAF6δ is dampened by inhibiting Notch signalling with GSI. Enriched pathways are shown, as are their associated P-values.

We further interrogated the TAF6δ-regulated genes whose induction was reduced by GSI. Of the 375 TAF6δ-regulated genes whose changes are dampened by inhibiting Notch signalling with GSI several classical Notch target genes were found including Hes1, Cyclin D1 and Dusp6 (Figure 20). Since our laboratory has recently shown that the mitochondrial BH3-only proteins Noxa and Bim are downstream effectors of TAF6δ-induced apoptosis (Delannoy et al., in preparation), we examined their expression in the data set. As expected, both the Noxa and Bim transcripts were induced by TAF6δ (Figure 20). The treatment with GSI of all of these Notch target genes resulted in a statistically significant reduction in their induction by TAF6δ (Figure 20), implying that the Notch pathway contributes to their increased expression. To provide further evidence that the Notch pathway is activated by TAF6δ, we examined the expression other established Notch target genes whose expression was induced by TAF6δ. We found that the induction of both the Notch target genes Hey1 (Maier and Gessler 2000) and Gata2 (Robert-Moreno et al. 2005) were statistically significantly reduced by GSI treatment (Figure 20). To control for specificity we also included two TAF6δ-regulated genes, Znf503 and Sesn2, whose expression was not reduced by GSI treatment (Figure 20). Taken together, our transcriptome analysis shows that inhibition of the Notch signalling pathway significantly dampens the induction a subset of TAF6δ-dependent genes that includes five previously identified direct Notch target genes.

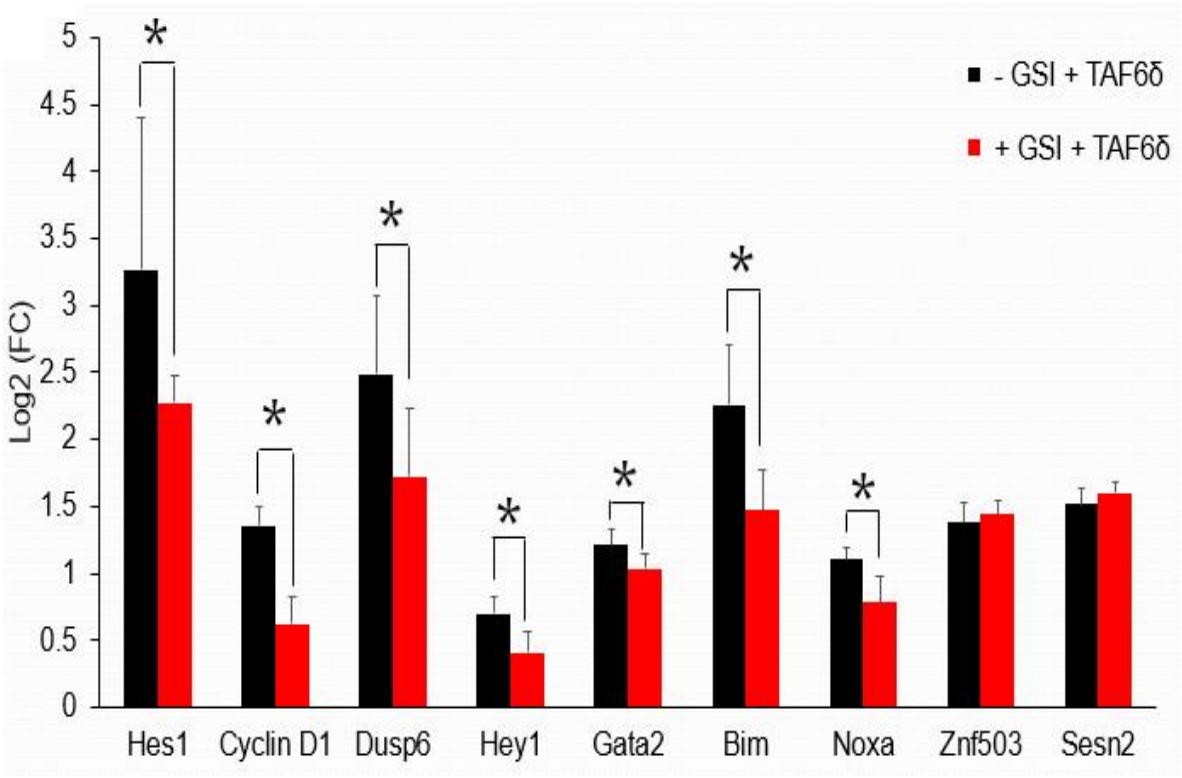


Figure. 20. TAF6δ-regulated transcription of Notch target genes. HeLa cells treated with GSI were transfected with SSO control or SSO TAF6δ, 18h post-transfection, RNA was extracted, and microarray analysis was performed. The fold induction, as measured by microarray analysis, of known Notch target genes and pro-apoptotic genes by TAF6δ is shown in the absence (black) and presence (red) of GSI (S2188). P <0.05*.

4.1.2 Effect of TAF6δ induction on genes of the Notch pathway at mRNA levels

To further validate and extend the analysis of the impact of TAF6δ on mRNA expression of genes from the Notch pathway, we performed qPCR on select genes from the Notch pathway in response to endogenous TAF6δ expression. qPCR experiments confirmed the induction of the classical Notch target genes Hes1 and Hey2 (Figure 21). We also examined the levels of the Notch ligands and found that Dll4 (Delta-like 4) was statistically significantly induced (Figure 21). The mRNAs of other ligands including Jag1, Jag2, Dll1 and Dll3 appeared to increase as well, though these changes did not reach statistical significance (Figure 21). We were particularly interested in the levels of the Notch receptors to obtain insight into possible mechanisms of Notch activation in response to TAF6δ. We found that Notch1 and Notch3 mRNAs were expressed at levels too low in HeLa cells to obtain reproducible quantitation

(Figure 21). In contrast, the mRNAs encoding Notch2 and Notch4 receptors were detectable (Figure 21). Interestingly, expression of the Notch2 mRNA was modestly but statistically significantly induced by TAF6δ (Figure 21). The qPCR confirm TAF6δ-dependent induction of several genes in the Notch pathway including the Dll4 ligand and Notch2.

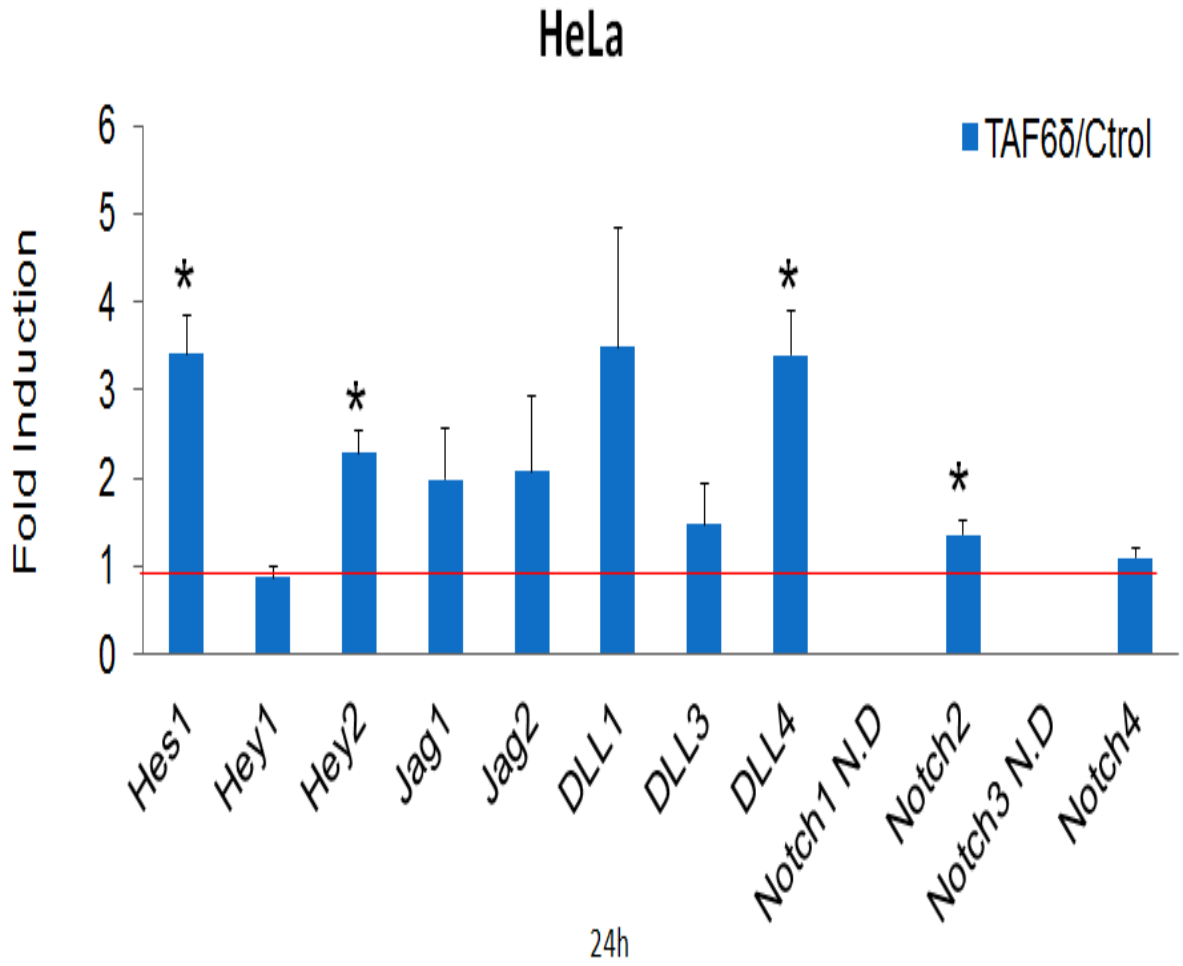


Figure 21. Effect of TAF6δ induction on Notch target genes at mRNA levels in HeLa cells. The expression of genes from the Notch pathway after 24h (except Dll4; 18h) transfection of SSOs, analysed by quantitative RT-PCR. Expression of hRPLPO was used as internal control and to normalize the RT-PCR data. The bars correspond to the ratio SSOs TAF6δ/ SSOs control. Data analysed by Student's t-test, P <0.05*. Error bars indicate the standard deviation of three independent experiments.

4.1.3 Effect of GSI on Notch pathway at mRNA levels after TAF6δ induction

We next tested the role of the Notch pathway in the TAF6δ-dependent transcriptional activation by employing GSI and qPCR to quantify mRNA levels. As seen with earlier microarray experiments, the expression of the Notch gene Hes1 was induced by TAF6δ and this induction was reduced in the presence of GSI (Figure 22). The induction of the Notch ligand Dll4 and the pro-apoptotic effector gene Bim by TAF6δ were also statistically significantly reduced (Figure 22). Reductions in Hey2 and Notch2 induction by TAF6δ were modest and were not statistically significant (Figure 22). Finally we tested a TAF6δ-dependent gene Acrc (Wilhelm et al. 2008) to control for the specificity of GSI treatment, since its expression was not reduced by GSI treatment (Figure 22). Overall, both the microarray experiments and our targeted qPCR revealed a selective set of TAF6δ-dependent genes whose induction is reduced by GSI treatment. The data suggest that Notch pathway activation can contribute to and shape the TAF6δ-triggered transcriptome landscape.

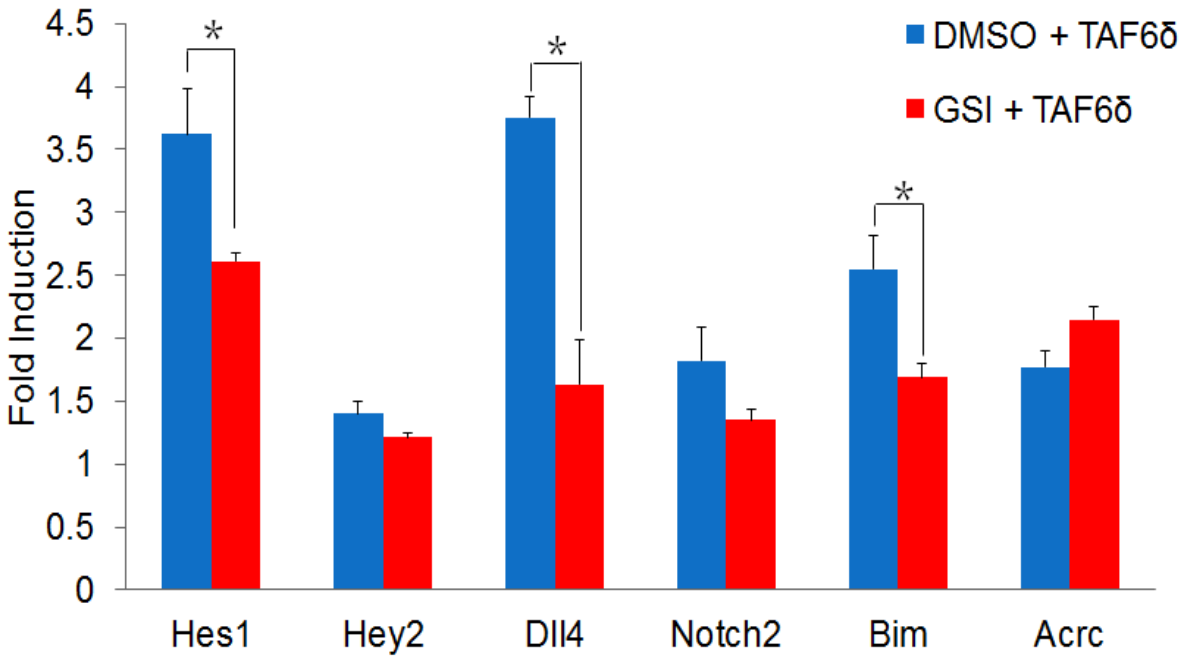


Figure 22. Effect of GSI on Notch target genes mRNA levels after TAF6δ induction in HeLa cells. Inhibition of the Notch genes expression after 18h (Hes1, Bim and Acrc) and 24h (Hey2, Dll4 and Notch2) treatment with 50μM of the GSI (DAPT) or DMSO as control for 1h before SSOs transfection, analysed by quantitative RT-PCR. Expression of hRPLPO was used as internal

control and to normalize the RT-PCR data. The y-axis represents the ratio of expression in SSOs TAF6δ treated cells versus SSOs control treated cells. Data analysed by Student's t-test, P <0.05*. Error bars indicate the standard deviation of three independent experiments.

4.2 Objective 2: Determine if TAF6δ affect Notch target genes expression in other cancer cell lines.

4.2.1 Effect of TAF6δ induction on Notch pathway genes in pancreatic and breast cancer cell lines

We next asked whether or not the activation of genes in the Notch pathway by TAF6δ occurs in cancer cells of other origins. In addition to HeLa cervical carcinoma cells, we induced endogenous TAF6δ expressing in pancreatic (Panc-1) cell line and two breast cancer cell lines (MDA-MB-231 and Hs-578-T) via transfection with SSOs. We surveyed genes within the Notch pathway by qPCR and found that indeed several Notch genes were induced in all three-cancer cell lines. Although certain genes were induced in a cell type specific fashion (for example Hey2, Figure 23), several genes were induced in all cell types tested including Hes1, Dll4, Notch2 and Bim (Figure 23). To control for the specificity of TAF6δ-induced gene expression we measured transcripts from the Hmox1 gene that displayed reduced or unaffected levels (Figure 23). In summary, the data show that the induction of Notch-related genes, including Hes1, Dll4, and Notch2, by TAF6δ is a general feature across several cancer cell types.

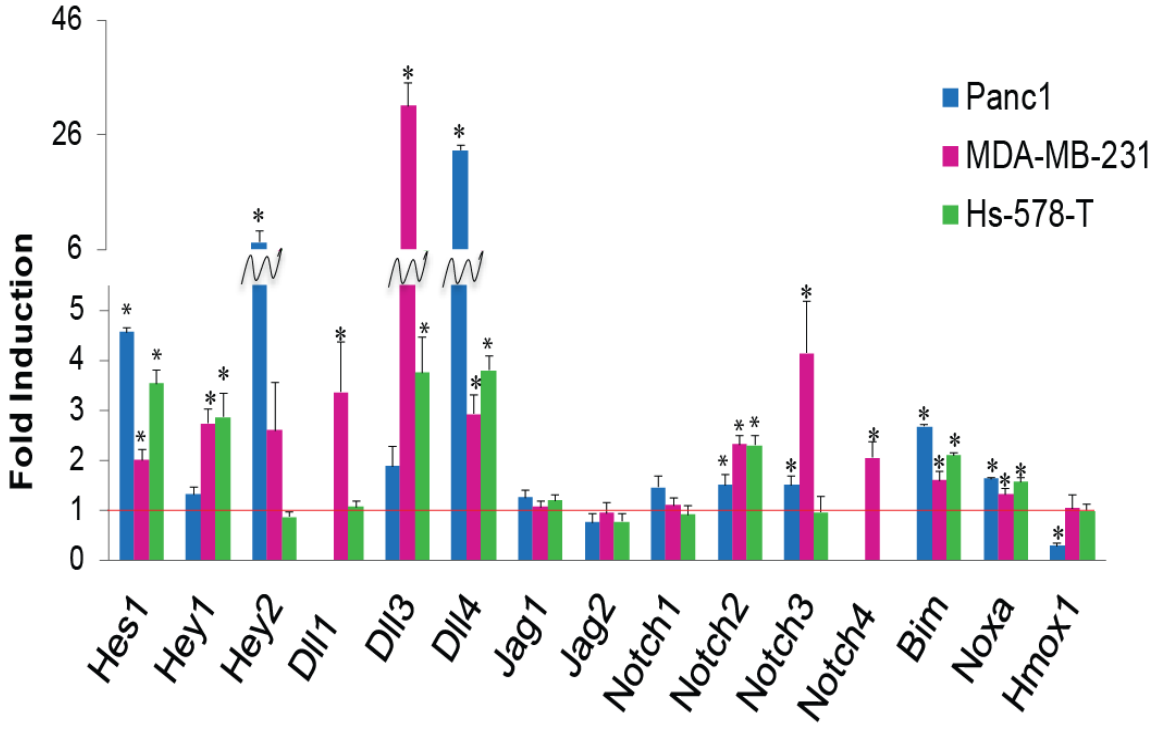


Figure 23. Effect of TAF6δ induction on Notch target genes at mRNA levels in different cancer cell lines. Notch ligands, receptors and target gene expression after 24h (except MDA 27h) transfection of SSOs, analyzed by quantitative RT-PCR. Expression of hRPLPO was used as an internal control and to normalize the RT-PCR data. The y-axis represents the ratio of expression in SSOs TAF6δ treated cells versus SSOs control treated cells. Data analysed by Student´s t-test, P <0.05*. Error bars indicate the standard deviation of three independent experiments.

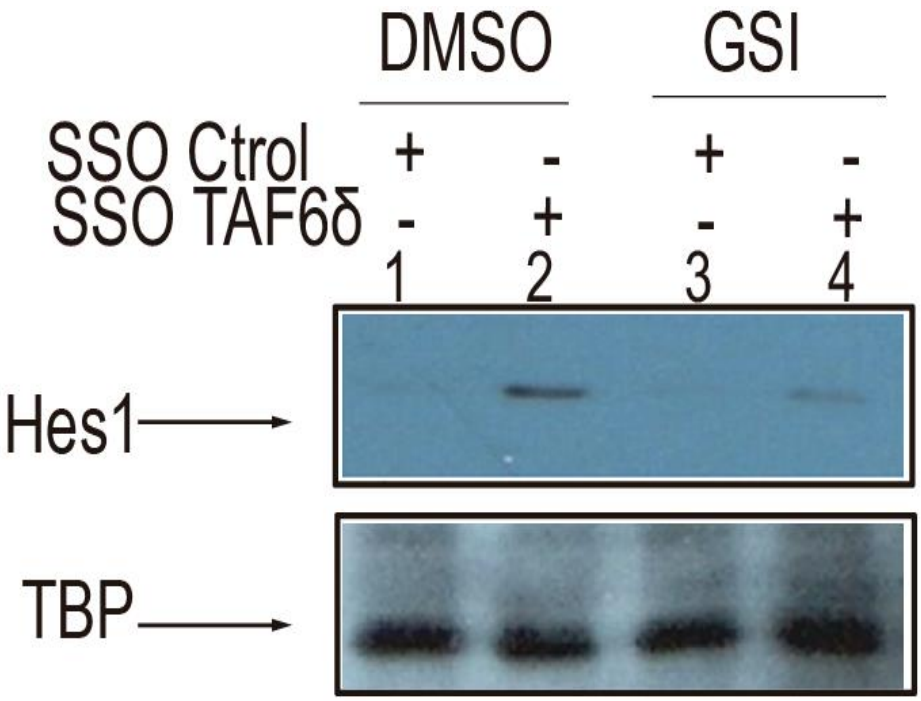
4.3 Objective 3: Test whether there is activation of the Notch pathway in response to TAF6δ in HeLa cells.

4.3.1 Effect of GSI on Hes1 protein levels after TAF6δ induction

The above microarray and qPCR analysis showed a γ-secretase-dependent induction of Notch target genes including Hes1 in response to TAF6δ at the mRNA level. To test if TAF6δ also increases the well-established Notch target Hes1 at the protein level, we used Western blot analyses to measure the expression of Hes1 protein in HeLa cells treated. We also tested

the requirement for Notch receptor cleavage by using GSI treatment. SSOs were transfected to induce TAF6δ expression followed by protein extraction and Western blot analysis. Western analysis with antibodies directed against Hes1 (Zhang et al. 2014) showed that the protein level of Hes1 was induced in response to TAF6δ (Figure 24A, lane 2 versus lane 1). Moreover, treatment with the GSI (DAPT), significantly decreased Hes1 induction by TAF6δ (Figure 24A & B). These findings show that the expression of a Notch pathway canonical target (Hes1) is affected by TAF6δ induction at the protein as well the mRNA level, and that this induction depends on γ-secretase activity. These results provide further support for the hypothesis that TAF6δ expression leads to the activation of the Notch signalling pathway.

(A)



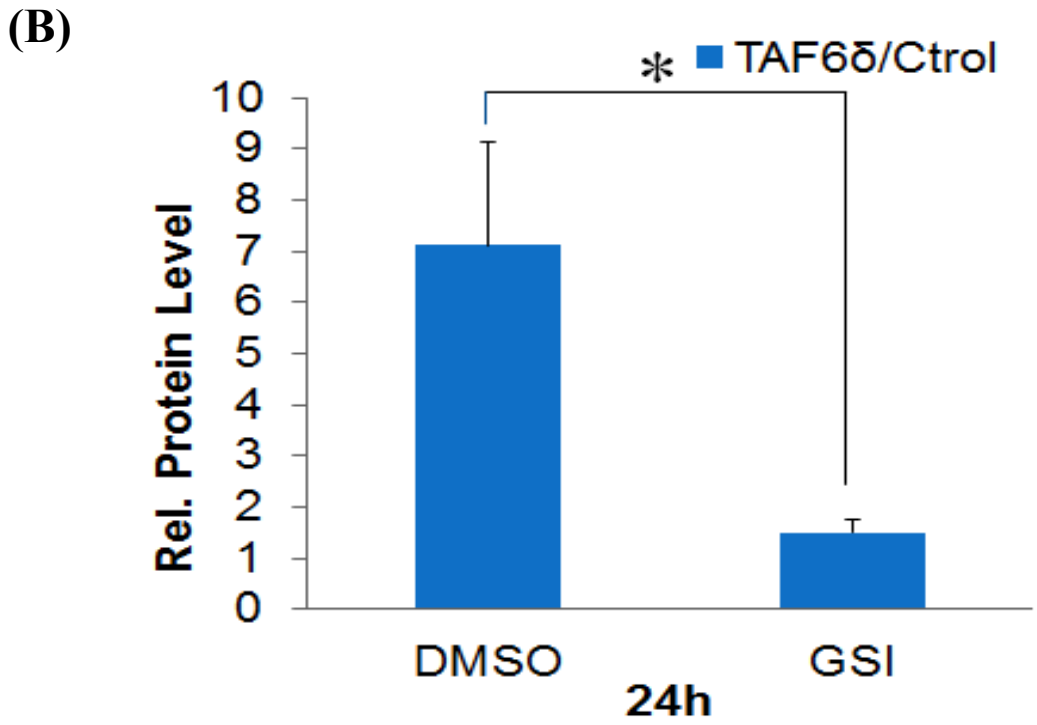


Figure 24. Effect of GSI on Hes1 protein levels after TAF6δ induction in HeLa cells. Inhibition of Hes1 protein expression after 24h treatment with 50µM of the GSI (DAPT) or DMSO as control for 1h before SSOs transfection, analysed by Western blot assays (A) Westerns blots were performed using an antibody against Hes1 (AB5702, Millipore; 1:2000), or TBP as a loading control. (B) Densitometry analysis of the bands corresponding to the ~37kDa Hes1 signal. Bands were quantified by ImageJ software. The values were normalized to TBP, the y-axis represents the ratio of protein expression in cells expressing TAF6δ versus control cells. Data were analysed by the Student's t-test, $P < 0.05$ *. Error bars indicate the standard deviation of three independent experiments.

4.3.2 Effect of GSI and EGTA on NIC-1 levels

Cleavage of the Notch receptors by γ-secretase is the crucial biochemical event leading to activation of the Notch receptor and its translocation to the nucleus. Notch1 is the best studied of the four receptors expressed in human cells. We took advantage of antibodies that specifically recognize the cleaved Notch1 intracellular domain (NIC-1) (Takam Kamga et al. 2016, Wang et al. 2016) to directly test whether the Notch1 receptor is activated in cells where TAF6δ expression is induced. Following SSOs induction of TAF6δ, we performed Western blot analysis with NIC-1 antibodies. No NIC-1 was detected in control HeLa cells (Figure 25, lane 1). Since we had previously shown that HeLa cells express only low levels

of Notch1 mRNA, we used the treatment with EGTA that induces Notch cleavage by chelating calcium. EGTA resulted in increased NIC-1 detection (Figure 25, lane 4 versus lane 2) and this increase was inhibited by GSI treatment (Figure 25, lane 5 versus lane 4). These results establish that the NIC-1 antibody can effectively detect cleaved NIC-1 from HeLa cell extracts.

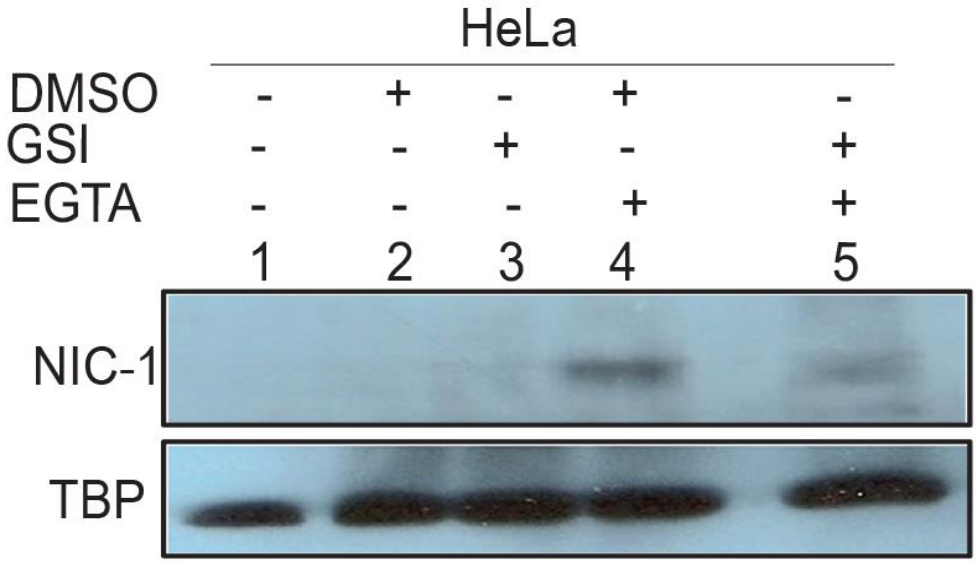


Figure 25. Effect of GSI and EGTA on the intracellular active domain of Notch1 (NIC-1) in HeLa cells. NIC-1 levels were analysed by Western blot with a specific antibody against the intracellular active domain NIC-1 (Cell Signalling D3B8, 1:1000), after 24h of treatment with 50µM of GSI (DAPT) or DMSO as control and 15 min treatment with EGTA followed by 1h recovery period. TBP was used as a loading control.

4.3.3 Effect of SSOs and EGTA on NIC-1 levels

To test the impact of TAF6δ induction on Notch1 activation, we transfected SSOs and followed cleaved NIC-1 levels by Western blot analysis. No detectable cleavage of Notch1 to yield NIC-1 in response to TAF6δ could be observed (Figure 26, lane 2). To ensure that the lack of NIC-1 was not due to the limits of detection of the antibody, we induced Notch1 cleavage to NIC-1 with EGTA treatment and again analyzed NIC-1 levels. EGTA treatment resulted in readily detectable NIC-1 (Figure 26, lane 3 versus lane 1), but these induced NIC-

1 levels were not further increased by the induction of TAF6δ (Figure 26, lane 4 versus lane 3). We conclude that Notch1 is not significantly activated in response to TAF6δ.

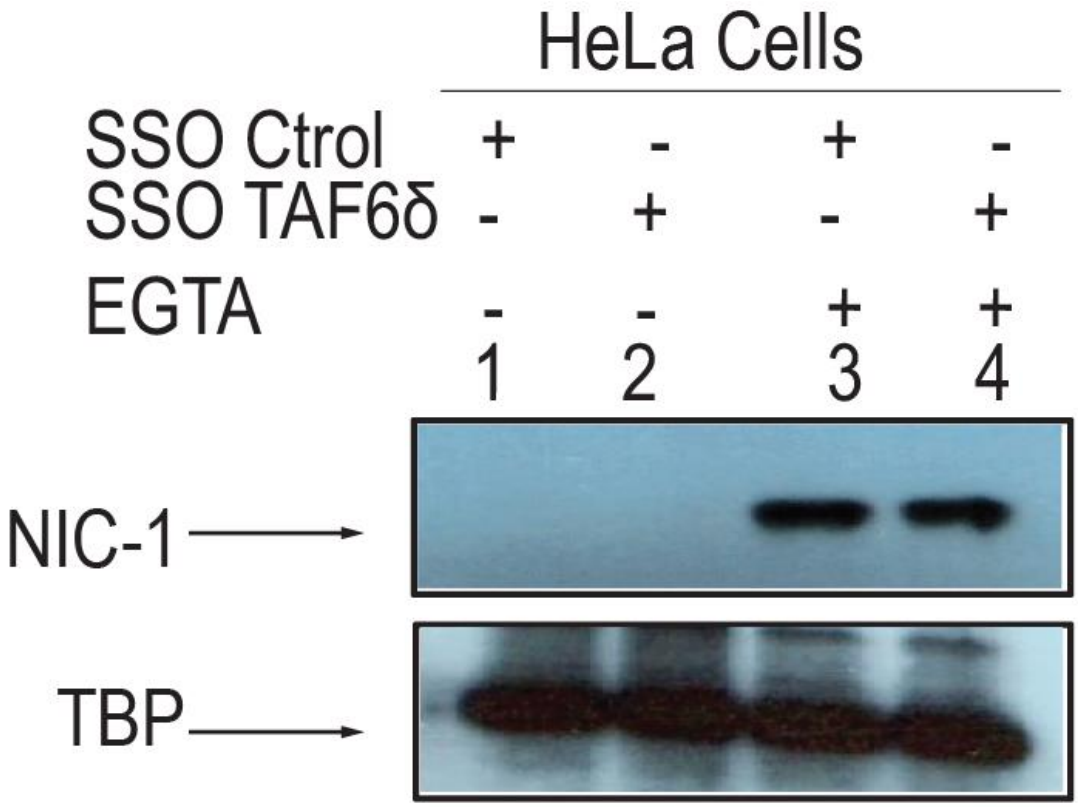
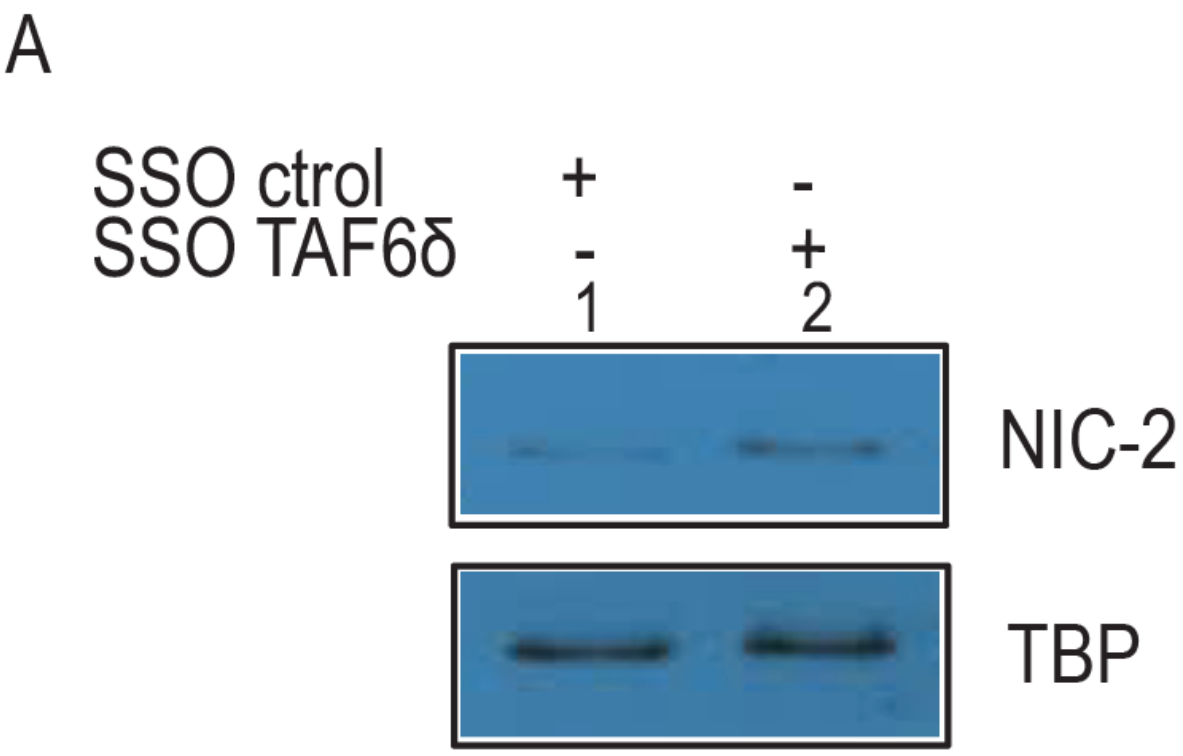


Figure 26. Effect of SSOs and EGTA on the intracellular active domain of Notch1 (NIC-1) in HeLa cells. Induction of NIC-1 was analysed by Western blot with a specific antibody against the intracellular active domain NIC-1 (Cell Signalling D3B8, 1:1000), after 24 h of transfection with SSOs and 15 min treatment with EGTA followed by 1h recovery period. TBP was used as a loading control.

4.3.4 Effect of TAF6δ induction on NIC-2 levels

Since we did not detect cleavage and activation of Notch1, we considered other Notch receptors as candidates for activation by TAF6δ. Notch2 was considered a promising candidate because, of the four Notch receptors, its mRNA is expressed at the highest levels in HeLa cells (Figure 21). More importantly, the Notch2 mRNA is induced in response to

TAF6δ (Figure 21). We used a monoclonal antibody that specifically recognizes the transmembrane/intracellular region, including the cleaved intracellular form of Notch2, NIC-2 (Gomi et al. 2015). NIC-2 has a diagnostic molecular weight of ~110 kDa, readily distinguishable from the full-length ~265 kDa Notch2 (Blaumueller et al. 1997). Endogenous TAF6δ induction via SSO transfection of HeLa cells caused an increase in the levels of NIC-2 (Figure 27A, lane 2 versus lane 1). Quantification of the results of three independent transfections by densitometry showed a statistically significant increase in the levels of NIC-2 in response to TAF6δ expression (Figure 27B). We conclude that TAF6δ expression in HeLa cells increases cleavage and activation of Notch2 to yield NIC-2.



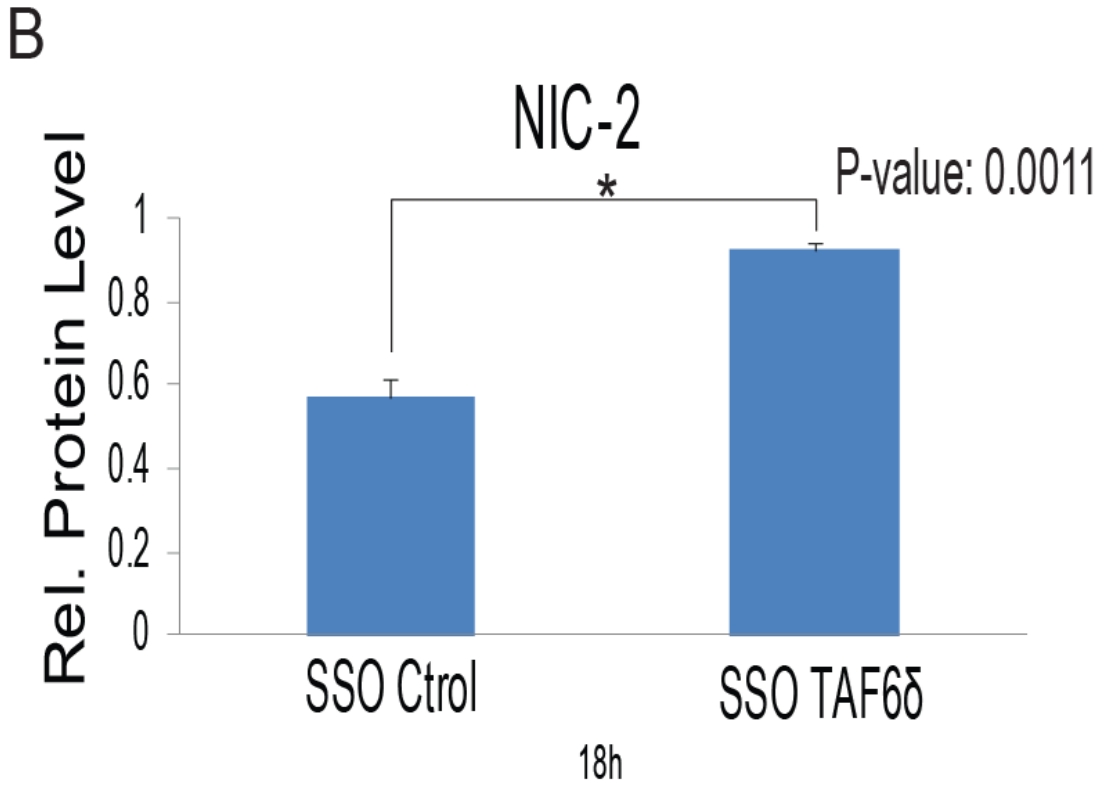


Figure 27. TAF6δ expression increases level of active Notch2 (NIC-2) in HeLa cells. Induction of NIC-2 after 18h of transfection with SSOs was analyzed by Western blot with an antibody against Notch2 (Cell Signaling D67C8, 1:1000) and a band was detected at the expected molecular weight of the cleaved fragment of Notch2 (A). (B) Densitometry analysis of the bands corresponding to the ~110kDa cleaved NIC-2 fragment. Bands were quantified by ImageJ software. The values were normalized to TBP, the y-axis represents the ratio of protein expression in cells expressing TAF6δ versus control cells. Data analysed by Student's t-test, P <0.05 *. Error bars indicate the standard deviation of three independent experiments.

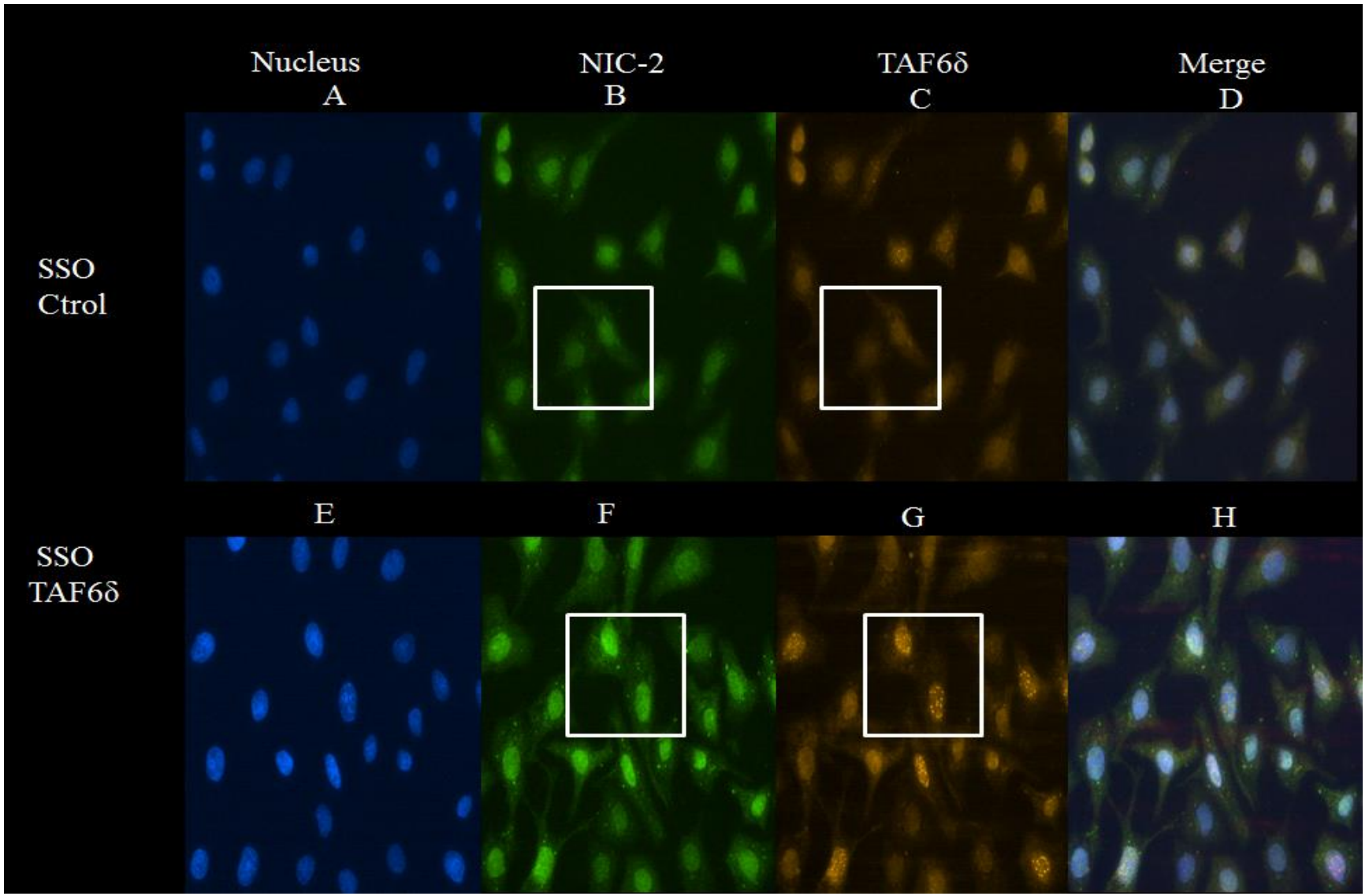
4.3.5 Effect of TAF6δ induction on cleaved NIC-2

To confirm NIC-2 cleavage as well as to determine whether NIC-2 activation occurs in cells expressing TAF6δ, we next performed immunofluorescence on HeLa cells with independently developed polyclonal antibodies that specifically recognize cleaved NIC-2 (Saravanamuthu et al. 2009). Double staining with antibodies directed against cleaved NIC-2 together with monoclonal antibody against TAF6δ was used to tract the expression of

TAF6δ in individual cells. As previously reported (Wilhelm et al. 2008), cells treated with TAF6δ-inducing SSOs induced punctate TAF6δ nuclear staining (Figure 28, panel F).
NIC-2 staining revealed both diffuse nuclear staining and discrete cytoplasmic foci that both appeared to increase in response to TAF6δ expression (Figure 28, panel F versus B). We interpret the nuclear staining as active (cleaved) chromatin-associated NIC-2 and interpret the cytoplasmic foci as active NIC-2 that has been released from the membrane to translocate towards the nucleus.

To quantify the immunofluorescence in an unbiased manner, we analyzed the images with the CellProfiler 2.1.1 algorithm (Broad Institute, Boston, USA). As expected, SSOs that induce TAF6δ expression resulted in a strong induction of the TAF6δ nuclear immunofluorescence signal (Figure 28, panel J). Quantification of the NIC-2 signal in cytoplasmic foci showed a statistically significant increase in the amount of cytoplasmic NIC-2 staining in response to TAF6δ (Figure 28, panel I). We quantified total nuclear NIC-2 staining as a measure of active NIC-2. TAF6δ-induction caused a statistically significant increase in nuclear NIC-2 signal (Figure 28; panel K). To further define the source of increased nuclear NIC-2 we calculated the percentage of increased signal (after removal of the background signal from control SSOs treated cells) found in TAF6δ positive versus TAF6δ negative cells. Interestingly, the majority (55%) of increased NIC-2 signal came from cells expressing TAF6δ (Figure 28, panel K), but a substantial portion of the increased signal also came from cells with no detectable TAF6δ (Figure 28; panel K). Taken in summary, the immunofluorescence data provide an independent confirmation that TAF6δ expression causes an increase in nuclear NIC-2, and further show that this increase occurs both TAF6δ-expressing and neighbouring cells. The results support a model wherein TAF6δ increases NIC-2 via intracellular signalling, but also via cell-cell signalling via the Notch pathway.

1



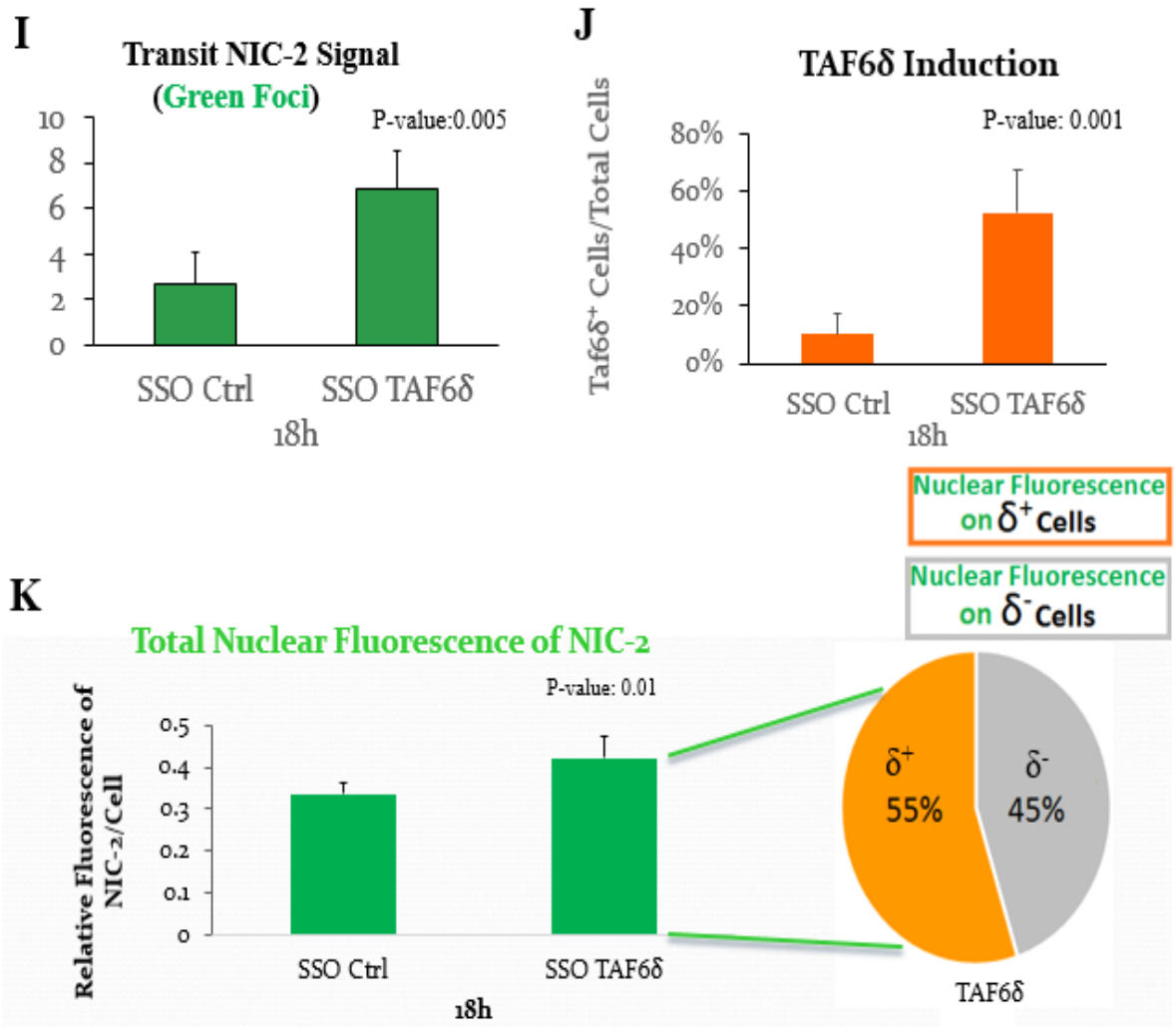


Figure 28. TAF6δ-expression increases level of active nuclear Notch2 (NIC-2) in HeLa cells. Induction of NIC-2 was analyzed by immunofluorescence with a specific antibody against the intracellular active domain NIC-2 (Abcam 8926, 1:1000), after 18h of transfection with SSOs (40x magnification in all panels). HeLa cells were treated with SSOs Control, panels (A-D) vs SSOs TAF6δ, panels (E-H). Panels (A and E) show nuclear staining with Hoechst. Panels (B and F), represent nuclear fluorescence of NIC-2, where two cells are indicated (rectangle). Panels (C and G) show TAF6δ expressing cells (nuclear orange foci), where two cells are indicated (rectangle). Panels (D and H) show the merge of the green and orange channels with the nucleus. (I) Quantitation of total green foci (Transit NIC-2 signal) counted divided by the total number of cells. (J) Quantitation of the percentage of TAF6δ induction calculated based on the number of cells with punctate TAF6δ staining/total number of cells. (K) Quantitation of the total nuclear fluorescence of NIC-2. The immunofluorescence was analyzed for at least 300 cells for each condition (SSOs Control and SSOs TAF6δ). Quantifications were performed by CellProfiler 2.1.1 software, P ≤0.01. Error bars indicate the standard deviation of four independent experiments.

B) General Objective

Can Notch activation contribute to TAF6δ-dependent apoptosis?

4.4 Objective 4: Determine the effect of inhibiting the Notch pathway on TAF6δ- induced apoptosis in HeLa cells.

4.4.1 Effect of GSI on apoptosis

Given that TAF6δ can induce Notch signalling via the activation of Notch2, we next tested the physiological impact of Notch signalling on TAF6δ-dependent cell death. We initially tested the effect of GSI (DAPT) treatment on apoptosis in normal culture conditions. Apoptosis was followed by flow cytometry using antibodies directed against activated Caspase-3. GSI treatment had no significant effect on the apoptosis observed under standard culture conditions of HeLa cells (Figure 29).

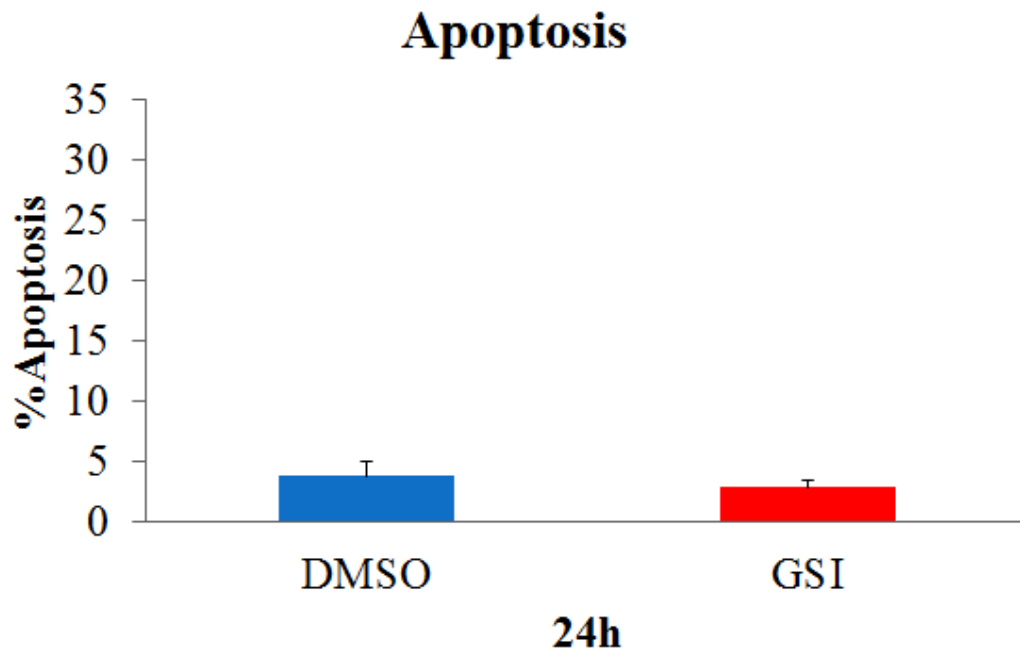


Figure 29. GSI does not affect apoptosis in HeLa Cells, as measured by flow cytometry. Cells were treated with GSI (DAPT) for 24h. The percentage of apoptotic cells was analysed by flow cytometry using a monoclonal antibody

that detects cleaved caspase 3. Error bars indicate the standard deviation of three independent experiments.

4.4.2 Effect of GSI on Cisplatin induced apoptosis

To further exclude the possibility of non-specific effects of GSI (DAPT) treatment in HeLa cells, we measured its impact on HeLa cells treated with the chemotherapeutic agent cisplatin. As before, we analyzed apoptosis by flow cytometry with antibodies against active Caspase-3. GSI caused no reduction in cisplatin-induced cell death at either of two concentrations used (Figure 30). We conclude that GSI does not non-selectively reduce apoptosis in HeLa cells.

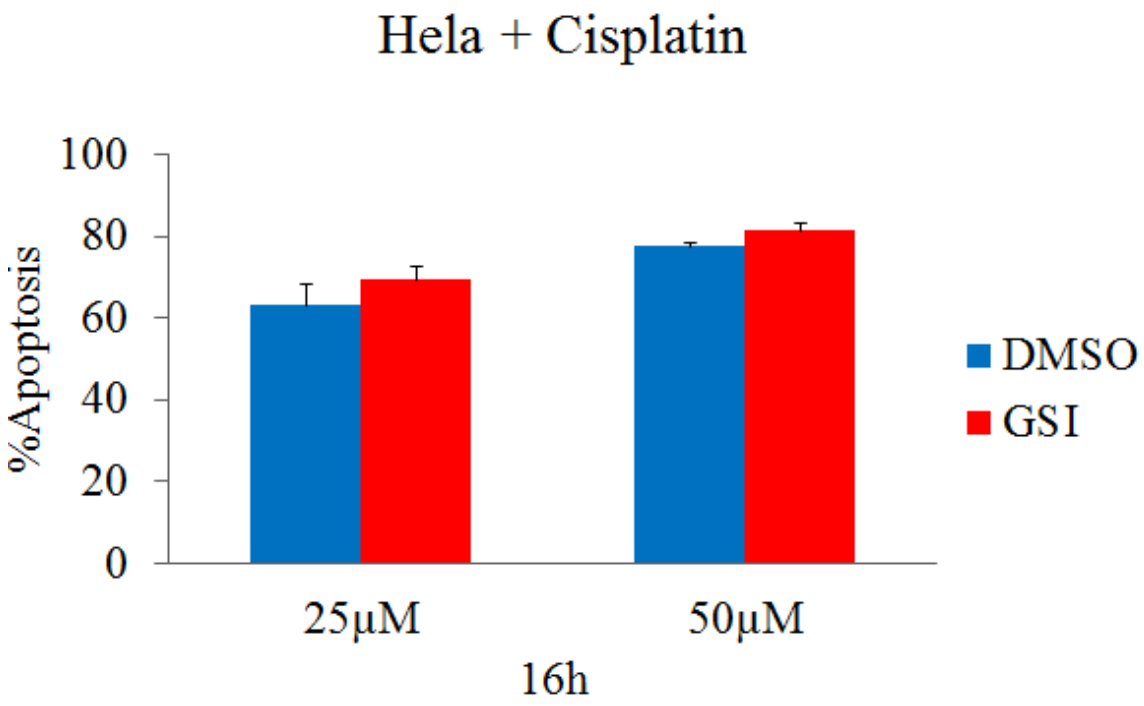


Figure 30. HeLa Cells in the presence of GSI and two doses of Cisplatin apoptosis-inductor. Cells were treated with 50µM of the GSI (DAPT) or DMSO as control for 1h, following by treatment with two doses of Cisplatin (25µM and 50µM) for 16h. The percentage of apoptotic cells was analysed by flow cytometry using a monoclonal antibody that detects cleaved caspase 3. Error bars indicate the standard deviation of three independent experiments.

4.4.3 Down-regulation of Notch signalling by GSI treatment reduces apoptotic effects of TAF6δ

We next investigated whether inhibition of Notch signalling by GSI treatment resulted in a significant reduction of apoptosis induce by TAF6δ expression in HeLa cells. We again performed flow cytometry to measure apoptosis by staining for cleaved caspase-3 (Neradil et al. 2015), a key protease in the execution of the apoptosis process (Riedl and Shi 2004, Parrish et al. 2013). GSI treatment caused a statistically significant drop in the levels of apoptosis induced by TAF6δ induction (Figure 31). These data demonstrate a specific reduction of TAF6δ induced apoptosis in presence of GSI, showing that Notch signalling contributes significantly to TAF6δ-driven apoptosis.

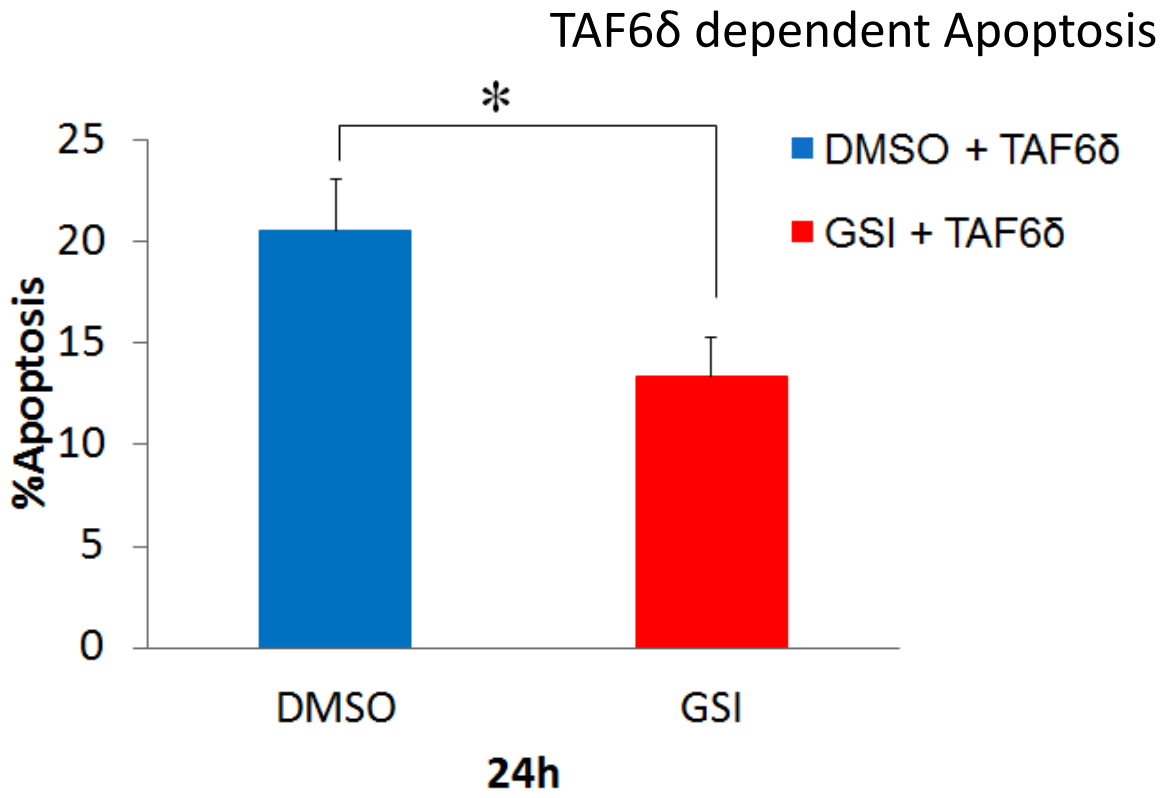


Figure 31. GSI reduces apoptotic effects of TAF6δ induction in HeLa Cells, as measured by flow cytometry. Cells were treated with 50μM of the GSI (DAPT) or DMSO as control for 1h, following by transfection of SSOs. Twenty-four hours later, the percentage of apoptotic cells was analysed by flow cytometry using monoclonal antibody that detects the functional cleaved caspase 3. The y-axis represents the ratio of apoptosis in SSOs TAF6δ treated cells versus SSOs control treated cells. Data analysed by Student's t-test, P <0.05*. Error bars indicate the standard deviation of three independent experiments.

5 DISCUSSION AND CONCLUSION

The Notch signalling pathway regulates several cellular processes, including cell differentiation, proliferation, and apoptosis. In mammalians, this pathway involves a group of Notch ligands (Delta-like and Jagged families) and Notch receptors (Notch 1-4) whose intracellular domain is translocated to the nucleus upon activation through cleavage by the γ-secretase complex. Once in the nucleus, the intracellular active domain (NIC) interacts with the CSL protein, activating the transcriptional complex that includes co-activators such as MAML1 and p300. Subsequently, it regulates the transcription of its Notch target genes: Hes1, Cyclin D1 and others (Table 5) (Kopan and Ilagan 2009, Andersson et al. 2011, Ranganathan et al. 2011, Schwanbeck et al. 2011, Wang 2011).

The TAF6δ pathway induces apoptosis (Wilhelm et al. 2008) and has also been implicated in the regulation of other pathways such as integrin, oxidative stress, angiogenesis and Notch (Wilhelm et al. 2010). However, the precise physiological context of TAF6δ-dependent cell death and the underlying molecular mechanisms remains unknown. In our previous study (Wilhelm et al. 2010), we found that endogenous TAF6δ induction has an impact on gene expression, including the induction of classic Notch target genes like Hes1, with the most significant P-value. By defining a mechanistic link between the Notch and TAF6δ signalling pathways, we hoped to acquire further knowledge into the specific physiological role of TAF6δ.

To test the hypothesis that TAF6δ activates Notch signalling and whether the Notch pathway participates in apoptosis induction, additional studies were needed. In this study, we investigated the association between TAF6δ and Notch pathways and their link with apoptotic process.

The goal of my master's work was, therefore, to answer two questions. Firstly, does TAF6δ expression cause activation of the Notch pathway? Secondly, can Notch activation contribute to TAF6δ-dependent apoptosis?

In order to answer the first question our first specific objective was to analyze the expression of Notch target genes in the presence and absence of GSI (γ-secretase inhibitor) after TAF6δ expression in HeLa cells. Our earlier results indicated that TAF6δ induction produces an increase in the expression of Notch target genes (Wilhelm et al. 2010). To verify whether inhibition of the Notch pathway with GSI (that prevents cleavage of full-length Notch to yield the active intracellular domain of Notch, NIC (Tolia and De Strooper 2009, Groth and Fortini 2012, Olsauskas-Kuprys et al. 2013)), affects gene expression in the presence of TAF6δ, we performed a microarray analysis that revealed that the GSI treatment selectively reduced TAF6δ-dependent induction of gene expression. We also observed that the analysis showed a significant decrease in the TAF6δ-dependent induction genes, including direct Notch down-stream target genes such as Hes1, Cyclin D1 and Dusp6 (Figure 19).

In addition, pro-apoptotic genes activated by TAF6δ like Bim and Noxa, which play an important role in apoptosis mediated by the intrinsic pathway (Ploner et al. 2008, Faber et al. 2012, Zhang et al. 2013). Bim, as a BH3-only protein is classified as an activator and has been implicated in the regulation of cell death when cells receive stimulus with growth factors and other stimuli like matrix detachment, cytokine deprivation, glucocorticoids and calcium deprivation. Moreover, Bim leads to apoptosis through the intrinsic pathway by activating Bax and Bak proteins (Sionov et al. 2015, Deng 2017). In addition, Noxa is also a BH3-only protein that is classified as a sensitizer due to its weak pro-apoptotic activity. Therefore, Noxa requires an partnership with other BH3-only members to be able to promote cell death (Albert et al. 2014).

In our microarray analysis, increased gene expression of Bim and Noxa; mediated by TAF6δ induction has also been shown to be prevented by Notch pathway inhibition (Figure 20). Our group has recently demonstrated that Bim and Noxa are effectors of TAF6δ (Delannoy et al., in preparation), therefore, one mechanism by which the Notch pathway may contribute to TAF6δ-driven apoptosis is by enhancing the expression of these pro-apoptotic genes. We also observed two genes that are not related to Notch pathway, Znf503 and Sesn2 in which the effect is different in presence of GSI. These results reinforce the selectivity of γ-secretase inhibitors, showing that only a subset of genes have been downregulated after blocking the

Notch pathway. These results are consistent with a role of the Notch pathway in the induction of TAF6δ-dependent apoptosis (Yang et al. 2004). We selected a group of genes and confirmed their regulation by qPCR experiments. First, we validated that genes belonging to the Notch signalling pathway were induced by TAF6δ, and indeed that Notch target genes, Notch ligands and specifically one Notch receptor (Notch2), displayed an increased expression in presence of TAF6δ (Figure 21). Subsequently, we tested the effect of inhibiting the Notch pathway with GSI in presence of TAF6δ. It was observed that the induction of Notch target genes as Hes1, Notch Ligands as Dll4, Notch2, and pro-apoptotic genes as Bim were reduced when the Notch pathway was blocked concomitantly TAF6δ induction with a significant P-value, $P < 0.05$ in HeLa cells (Figure 22). Hence, these results corroborate our microarray data and suggest a link between TAF6δ and Notch pathways in HeLa cells.

Our second specific objective was to determine if TAF6δ affects Notch target gene expression in other cancer cell lines. We found that TAF6δ expression is able to up-regulate various Notch target genes, Notch ligands mostly Delta-like family, Notch receptors and pro-apoptotic genes in pancreatic (Panc-1) and breast (MDA-MB-231 and Hs-578-T) cancer cell lines as it was shown above with the cervical cancer cell line, HeLa (Figure 23). Therefore, the microarray and qPCR data shows that TAF6δ can drive the expression of genes in the Notch pathway in several independent cancer cell lines of distinct tissue origins.

After, having shown that a subset of TAF6δ-induced genes is blocked upon Notch pathway inhibition (using GSI), we proceeded to determine the level of Notch activation upon TAF6δ expression in HeLa cells.

Hes1 is the most well characterized Notch target gene that can regulate cell fate decisions (Fischer and Gessler 2007, Ranganathan et al. 2011, Liu et al. 2015). Hes1 functions include cell cycle control that create a balance within proliferation and differentiation (Monahan et al. 2009) and an important role in development of the nervous system, pancreas and lymphocytes (Fischer and Gessler 2007). It has also been published that Hes1 mediates the connection between Notch pathway and apoptosis (Nickoloff et al. 2005, Robert-Moreno et al. 2007, Konishi et al. 2010, Kannan et al. 2011).

In addition to demonstrating increases in Hes1 mRNA levels, we also wanted to know at the protein level whether the inhibition of the Notch pathway resulted in decreased expression of this classical Notch target gene in response to TAF6δ induction. Indeed, we found that TAF6δ-induced Hes1 expression was significantly reduced in GSI presence (Figure 24), which strongly suggest that TAF6d induces Hes1 transcription through activation of the Notch pathway in HeLa cells.

Furthermore, our study also showed that TAF6δ does not increase the level of the cleaved Notch1 intracellular domain (NIC-1). In order to observe NIC-1, EGTA (calcium-chelating) was added to induce the cleavage of the Notch 1 (Rand et al. 2000) (Figures 25-26). EGTA induces Notch1 cleavage as this receptor is composed of two domains, the extracellular and the intracellular, that are held together via non-covalent interactions that depend on calcium. Therefore, calcium depletion results in the dissociation of the extracellular and intracellular domains so the intracellular domain became more accessible to proteolysis by γ-secretase complex (De Strooper et al. 1999, Rand et al. 2000). Given that TAF6δ did not lead to increased NIC-1 expression, this result suggested an activation of the Notch pathway by TAF6δ through another receptor, like Notch2. Increased cleavage of Notch2, the only receptor upregulated by TAF6δ, was increased upon TAF6δ expression as measured by western blot and qPCR (Figure 27). This result suggests a direct activation of the Notch signalling pathway by TAF6δ expression through the Notch2 in HeLa cells. We did not test Notch3 and Notch4 receptors in HeLa cells. Due to the qPCR results revealed that Notch3 levels were too low to be able to detect and Notch4 were not significantly affected by TAF6δ. The demonstration of Notch2 cleavage in response to TAF6δ provides the first mechanistic information underlying the functional link between the TAF6δ and Notch pathways.

In order to confirm whether TAF6δ activates the Notch2, nuclear NIC-2 levels were measured after TAF6δ induction (Figure 28). Immunofluorescence analysis showed good transfection efficiency, with 53% of cells induced to express TAF6δ (P-value, $P < 0.05$). The analysis also showed that total NIC-2 nuclear fluorescence increased in TAF6δ presence with a statistical P-value, $P < 0.05$ and the contribution of this fluorescence came mainly from cells

expressing TAF6δ with 55%, implying that Notch2 activation leading to NIC-2 nuclear localization occurs in TAF6δ–expressing cells.

Nevertheless, there was a contribution (45%) from cells not expressing TAF6δ. These data are consistent with a model wherein both intracellular signalling, via transcriptional crosstalk between Notch2 and TAF6δ, and extracellular signalling, via Notch ligand induction, contribute to enhance apoptosis. This model is further supported by our microarray, qPCR and western blot experiments that demonstrated the induction of both Notch2 cleavage and increased Notch ligand expression (eg. Dll4) in cell populations where endogenous TAF6δ is enforced. Activation of Notch2 has been reported through ligand-expressing cells (Dll1-4 and Jagged1-2) by co-culture or recombinant Dll4 that also required a proteolytic cleavage through Adam10 metalloprotease and γ-secretase complex to allow transcriptional activation of the Notch target genes (Groot et al. 2014). The physical binding of the Notch ligands (Delta-like and Jagged1-2) to the endogenous Notch2, with higher affinity for the Delta family (eg. Dll1) had been validated by a cell-cell association assay. This Dll1-Notch2 association, in order induces cleavage and nuclear translocation of NIC-2 (Shimizu et al. 2000).

Moreover, another parameter evaluated was total NIC-2-specific fluorescence foci in the cytoplasm in the presence and absence of TAF6δ. Our interpretation of these foci is that they represent NIC-2 in transit, while active NIC-2 is translocated from the cytoplasm to the nucleus. The measures revealed that when TAF6δ is induced, we counted more NIC-2-specific foci in the cytoplasm. We interpret the results as implying that in presence of TAF6δ there is more active NIC-2 in transit to the nucleus. Our results also confirmed by immunofluorescence that the expression of TAF6δ induces an increased the active Notch2 within the nucleus.

These results suggest that cell to cell communication contribute to Notch activation in neighboring cells, a model that is consistent with the well-established role of Notch signalling between cells (Kato 2011). Cellular communication studies showed that the Lunatic fringe protein (Lfng) is able to potentiate both ligands Jagged1 and Delta-like1 signalling towards

Notch2 (Hicks et al. 2000) but inhibits Jagged1 and potentiates Delta-like1 signalling through Notch1. These reports suggest that Lfng modulates the interactions between Notch ligands and Notch receptors, indicating that there is a positive feedback loop from cell-to-cell to generate signal propagation (Hicks et al. 2000, Matsuda et al. 2012). In fact, another study demonstrated that endothelial cells communicate with mural cells in order to regulate vessel assembly and differentiation through Notch3 activation by a positive-feedback loop that includes autoregulation and Jagged expression (Liu et al. 2009).

To determine whether Notch activation can contribute to TAF6δ-dependent apoptosis, our fourth specific objective was to determine the effect of inhibiting the Notch pathway on TAF6δ-induced apoptosis in HeLa cells. It has been reported that the activation of the Notch pathway is capable of contributing to the induction of apoptosis, such as tumor suppressor (Chadwick et al. 2008, Jiao et al. 2009, Nowell and Radtke 2017). Indeed, it has been shown that overexpression of Notch1 leads to inhibition of proliferation followed by apoptosis through up-regulation of p21 (promotes cell cycle arrest) (Nowell and Radtke 2017), IFI 16 (inhibit cell cycle and induce apoptosis) (Chadwick et al. 2008), caspases 3, 9 and down-regulation of cyclin E, Cdk2 and bcl-2 (Jiao et al. 2009). In addition, ablation of Notch activity through deletion of Notch1 and Notch2 or CSL increased tumor development, indicating that Notch can act in an anti-tumorigenic manner (Nowell and Radtke 2017). Another contribution to this network between the Notch and apoptosis pathways comes from the most studied gene of the Notch signalling, Hes1. The functions described to contribute to the ability of Hes1 to induce apoptosis include: its association with PARP1 protein (Kannan et al. 2011), negative regulation of the anti-apoptotic proteins of the Bcl-2 family (Robert-Moreno et al. 2007) and the induction of the pro-apoptotic BH3-only proteins, Bim and Noxa (Nickoloff et al. 2005, Konishi et al. 2010).

To test a role of Notch signalling in TAF6δ-dependent apoptosis, we initially tested whether GSI could non-specifically affect the percentage of apoptosis. Flow cytometry measures did not reveal significant differences in the percentage of apoptosis between treatment with GSI and DMSO (inhibitor vehicle) in HeLa cells under standard growth conditions (Figure 29). Subsequently, a positive inducer of apoptosis, cisplatin, was tested in combination with GSI,

but no significant differences were observed between percentages of apoptosis induce with DMSO vs GSI (Figure 30). Having determined that the GSI did non-selectively reduce apoptosis, we wanted to test the effect of GSI specifically on TAF6δ-mediated cell death. We asked if down-regulation of the Notch pathway could result in a reduction of apoptosis induction by TAF6δ. The results revealed that down-regulation of Notch signalling reduced TAF6δ-dependent apoptosis levels by 17% (Figure 31). This suggests that Notch pathway activation by TAF6δ positively influences the apoptotic cascade. This result, to my knowledge, is the first example of reciprocal feedback between the Notch pathway and apoptosis. In other words, pro-apoptotic TAF6δ activates Notch signalling, that in turn contributes to the process of apoptosis. Based on the direct link between Notch target genes expression and TAF6δ, we propose a model in which activation of the Notch pathway by TAF6δ is essential for initiating TAF6δ-mediated apoptotic process (Figure 32).

Further work will be required to determinate which specific molecular mechanisms TAF6δ exploits to induce Notch pathway activation. One hypothesis could be that TAF6δ could induce the expression of one of the Notch ligands in order to activate the Notch receptor as reported in the literature (Wang 2011), like Delta-like four (Dll4), for example. Dll4 has been associated with cervical cancer (Yang et al. 2016) and was induced in all the cancer cell lines that we tested (Figure 21 and 23). Therefore, we propose a crosstalk model that shows an activation of the Notch signalling pathway through Delta-like four ligand by TAF6δ and its association contributes to apoptosis in HeLa cells (Figure 33).

Another possibility could be an association between TAF6δ and the γ-secretase complex, (which increases Notch intracellular domain release by the γ-secretase cleavage (Jurisch-Yaksi et al. 2013)) based on our microarray data that showing increased expression in some subunits of this complex, like Aph1b (Anterior pharynx-defective 1) and Pen2/Psenen (Presenilin Enhancer 2). However, to validate this hypothesis, additional studies will be needed. Indeed, the characterization of the complete molecular mechanism through which TAF6δ activates the Notch pathway should provide a better understanding the complex interplay between TAF6δ and cell death, particularly in the context of tumor biology.

In terms of perspectives, it would be interesting to study whether other cancer cell lines where TAF6δ induces apoptosis use the same molecular mechanism found in HeLa cells, in which TAF6δ, the Notch signalling pathway and the apoptosis process are associated. In addition, it will be important to confirm activation of the intracellular domain of Notch2 (NIC-2) and the cellular localization of NIC-2, in the absence and presence of GSI after TAF6δ expression in HeLa cells. Another parameter to evaluate could be to verify whether TAF6δ regulates the expression of the different subunits of the gamma-secretase complex. To confirm the involvement of Notch2 activation in the TAF6δ-induced effect, it would be mandatory to assess the impact of knocking down Notch2 in TAF6δ-expressing cells. As well, it should be fascinating to analyze in a physiological context whether TAF6δ-Notch crosstalk interacts functionally with other pathways that were statistically over-represented after TAF6δ induction in microarray analysis, such as hypoxia or angiogenesis (Wilhelm et al. 2010). It has been reported in the literature a link between Notch and hypoxia pathways through the regulation of Notch ligands, Notch receptors or the transcriptional activation of Notch downstream target genes such as Hes1 (Lee et al. 2009, Borggrefe et al. 2016). The first molecular mechanism of action that were proposed elucidated an up-regulation of the Notch ligand, Dll4 by hypoxia in a HIF-1α-dependent manner (Borggrefe et al. 2016). In addition, the Notch pathway has been implicated as an important regulator of angiogenesis. Notch signalling is able to regulate the differentiation of endothelial cells and blood vessels, but defects in Notch can lead to inhibition on network formation of the endothelial cells and vessel-like structures in angiogenesis, and also lead to cardiovascular diseases (Liu et al. 2003, Gridley 2007).

In summary, the results presented here demonstrate that inhibition of the Notch pathway reduces the TAF6δ-dependent induction of Notch ligand (Dll4) and Notch target genes. In addition, several Notch target genes, Notch ligands and Notch receptors are upregulated by TAF6δ expression in different cancer cell lines. Furthermore, TAF6δ activates the Notch signalling pathway through cleavage of the Notch2 and is involved in the induction of apoptosis in HeLa cervical cancer cell line. Finally, the data demonstrated that activation of the Notch pathway by TAF6δ contributes to TAF6δ-driven apoptosis.

A model for crosstalk between the TAF6δ and Notch pathways

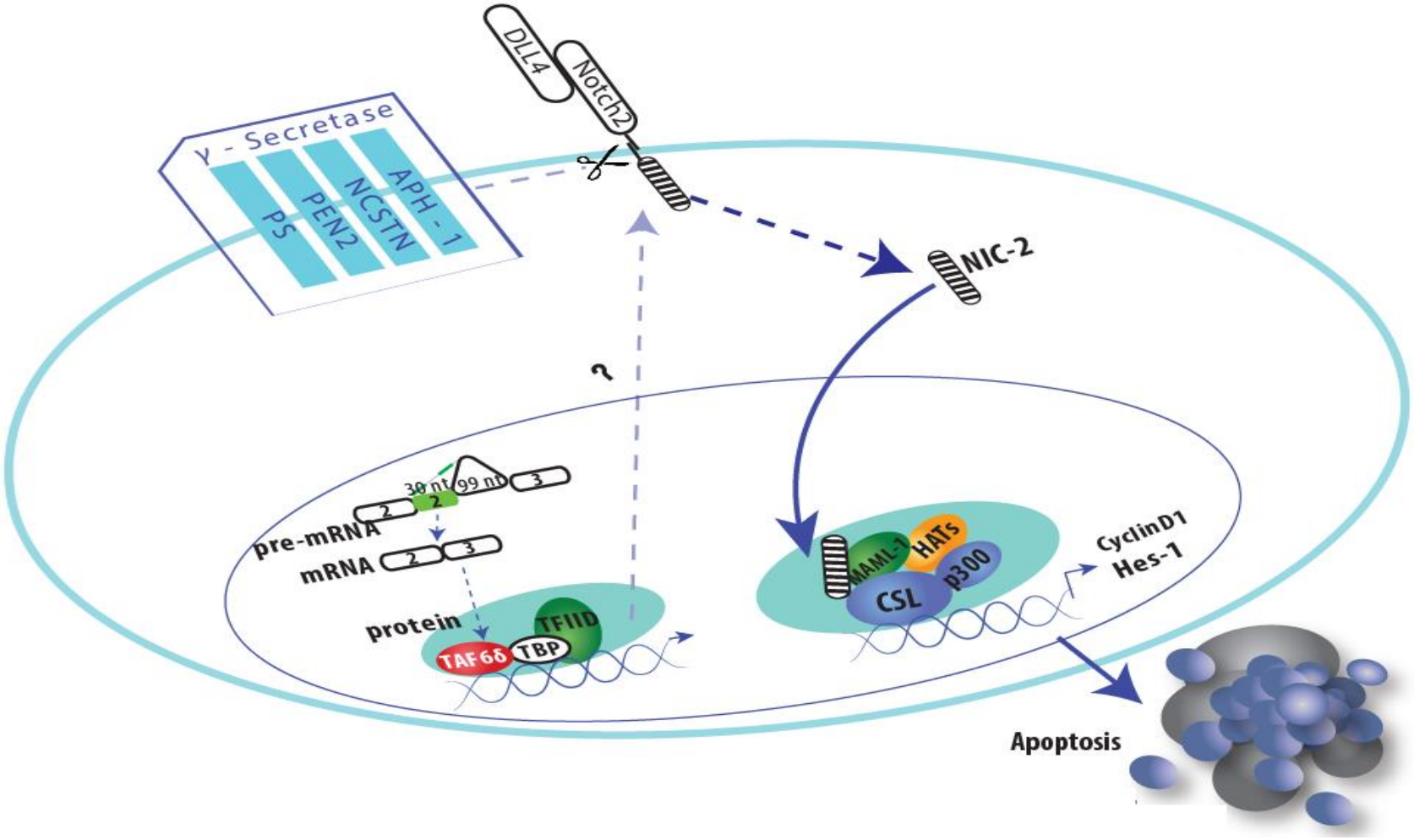


Figure 32. First model for crosstalk between the TAF6δ and Notch pathways. A hypothetical model showing the activation of Notch pathway by TAF6δ through activation of Notch2 (see text for details).

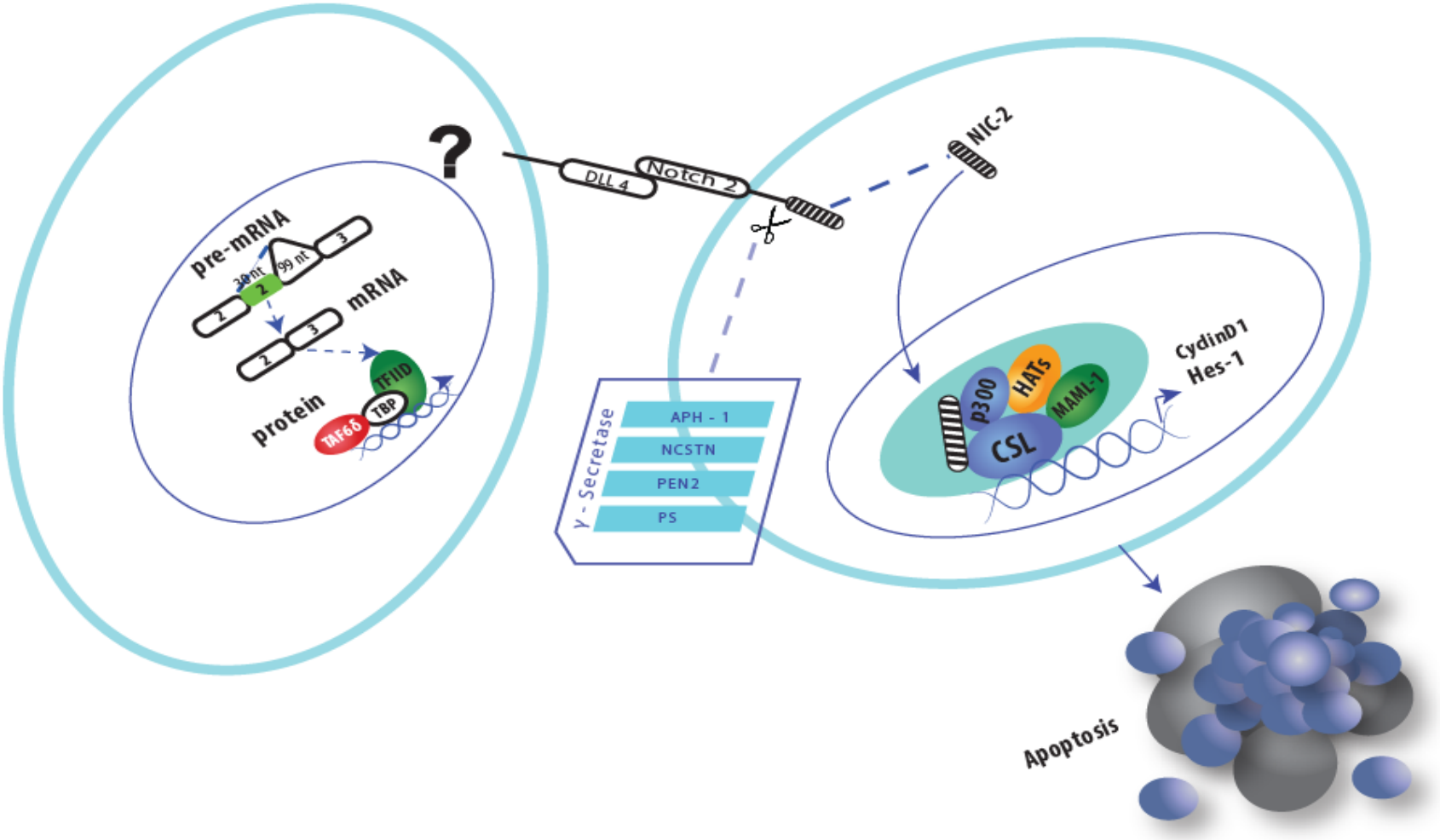


Figure 33. Second model for crosstalk between the TAF6δ and Notch pathways. A hypothetical model showing the activation of Notch pathway by TAF6δ through activation of Notch ligand, Dll4 (please see text for details).

6. ATTACHMENTS

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Attachment 2

Table 5. Notch Signalling Pathway Target Genes

Cellular Process	Notch Target Genes
Apoptosis	CDKN1A, CFLAR (CASH), IL2RA, NFKB1
Cell Cycle Regulators	CCND1, CDKN1A, IL2RA
Cell Proliferation	CDKN1A, ERBB2, FOSL1, IL2RA
Cell Differentiation Regulators	DTX1, PPARG
Neurogenesis	HES1, HEY1
Regulation on Transcription	DTX1, FOS, FOSL1, HES1, HEY1, NFKB1, NFKB2, NR4A2, PPARG, STAT6
Other Target Genes with Unspecified Functions	CD44, CHUK, IFNG, IL17B, KRT1, LOR, MAP2K7, PDPK1, PTCRA. CCND1, CDKN1A, GATA3 and PTCRA

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