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Post-transcriptional control of TP63 isoforms by PTBP1 in Head and Neck Squamous Cell Carcinoma

William Taylor

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L'UNIVERSITE DE RENNES 1

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Biologie Santé

Spécialité : « *Cancérologie* »

Par

« **William TAYLOR** »

« **Régulation post-transcriptionnelle des isoformes de TP63 par la PTBP1 dans les carcinomes épidermoïdes de la tête et du cou** »

Thèse présentée et soutenue à Rennes, le 26 Novembre 2021

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Table of Contents

ABBREVIATIONS.....	
INTRODUCTION.....	
A <i>TP63</i> centric view of the <i>TP53</i> family.....	
I. The <i>TP53</i> family, an evolutionary toolbox for genome and tissue maintenance and integrity.....	
II. Three genes, many more isoforms.....	
III. Molecular functions and regulations of p63.....	
III.1. Different functions among different isoforms.....	
III.1.1. TAP63 vs Δ NP63.....	
III.1.2. C-terminal isoforms.....	
III.2. Regulation of <i>TP63</i> expression.....	
III.2.1. Transcriptional regulation of TP63.....	
III.2.2. mRNA stability.....	
III.2.3. Protein stability and degradation.....	
III.2.4. miRNAs targetting TP63.....	
IV. p63 developmental functions.....	
IV.1. p63 in the establishment of the epidermis.....	
IV.2. p63 regulates many other epithelia.....	
IV.2.1. p63 in the eye: corneal regeneration.....	
IV.2.2. p63 in the uterus and vagina.....	
IV.2.3. p63 in mammary tissue development and maintenance.....	
IV.3. p63 in non-epithelial tissue developmental.....	
IV.3.1. Limbs/bones.....	
IV.3.2. Muscle.....	
IV.3.3. Heart.....	
IV.3.4. Cochlea.....	
V. p63 in germline protection.....	
VI. Pathological involvement of p63:.....	
VI.1. p63 associated human syndromes.....	
VI.2. Cancer.....	
VI.2.1. p63 as a biomarker.....	
VI.2.2. Tumour suppressor and oncogenic roles of p63.....	
Maturation of pre-mRNA and its actors.....	
I. Maturation of pre-mRNA.....	
I.1. Correct recognition of exons and intron boundaries requires specific sequences in specific places.....	
I.2. The challenge of large introns.....	
I.3. The spliceosome: where the magic happens.....	
I.4. Cleavage and polyadenylation.....	
II. Alternative splicing, alternative cleavage and polyadenylation, and their regulation.....	
II.1. Alternative splicing.....	
II.2. Alternative cleavage and polyadenylation.....	
II.3. Regulatory factors.....	
II.3.1. The SR protein family.....	
II.3.2. The hnRNP protein family.....	
II.4. Functional and pathological results of aberrant splicing.....	
II.5. Therapeutic strategies to modulate pathological splicing.....	
III. Focus on the polypyrimidine tract binding protein (PTBP1) hnRNP.....	
III.1. Role in translation.....	

III.2. PTBP1's role in pre-mRNA splicing.....
III.3. <i>PTBP1's role in RNA polyadenylation</i>
III.4. PTBP1 and Cancer.....
Cancer, splicing and the EMT.....
AIMS OF THE STUDY.....
RESULTS.....
Chapter 1. Functional characterisation of the involvement of the RNABP PTBP1 in the regulation of <i>TP63</i> isoform production.....
1.1. Article: "PTBP1 controls <i>TP63</i> alternative splicing in HNSCC tumour cells".....
1.2. "PTBP1 controls <i>TP63</i> alternative splicing in HNSCC tumour cells": Discussion and perspectives.....
Chapter 2. Investigation of <i>TP63</i> isoform function in human epithelial cells.....
2.1. Comparison of EMT-related phenotypes between PTBP1 depleted and TGF- β treated HaCaT cells.....
2.2. Comparison of EMT-related phenotypes between PTBP1 depleted and TGF- β treated SCC-9 cells.....
2.2. Identifying p63 targets in HNSCC cells.....
2.3. Using <i>TP63α</i> and <i>TP63γ</i> overexpressing SCC-9 cells to identify differential targets and functions.....
Chapter 3. Development of monoclonal antibodies targeting specific isoform of <i>TP63</i>
3.1. Mice immunisation and control.....
3.2. Hybridoma generation.....
3.3. Assessing the sensitivity and specificity of the purified antibodies.....
3.4. Conclusion.....
Chapter 4. Identification of circRNAs derived from the <i>TP63</i> gene in head and neck squamous cell carcinoma (HNSCC).....
4.1. Identifying <i>TP63</i> circRNAs in HNSCC cell lines.....
4.2. Does PTBP1 influence <i>TP63</i> 's back-splicing into circRNAs?.....
4.3. Conclusion.....
GENERAL DISCUSSION.....
BIBLIOGRAPHY.....
RÉSUMÉ FRANÇAIS.....
I. Contexte bibliographique de la thèse.....
II. Situation du sujet de la thèse.....
III. Résumé des résultats obtenus lors de la thèse.....
IV. Conclusion et perspectives ouvertes par ce travail.....

ABBREVIATIONS

AER: Apical Ectodermal Ridge
AGEZ: AG Exclusion Zone
APA: Alternative PolyAdenylation
AS: Alternative Splicing
BLM: Bleomycin
BPS: Branch Point Sequence
CBC: Cap Binding Complex
CF Im: Mammalian cleavage factor I
CF Iim: Mammalian cleavage factor II
ChIP-seq: Chromatin Immunoprecipitation Sequencing
circRNA: circular RNA
CK: CytoKeratin
CTC: Circulating Tumour Cell
CTD: C-terminal Domain of RNA-polymerase II
CPSF: Cleavage and Polyadenylation Specificity Factor
CstF: Cleavage stimulation Factor
DBD: DNA Binding Domain
DSE: Downstream Sequence Element
EJC: Exon Junction Complex
EMT: Epithelial to Mesenchymal Transition
ESE: Exonic Splicing Enhancer
ESS: Exonic Splicing Silencer
FGF: Fibroblast Growth Factor
HDAC: Histone Deacetylase
HNSCC: Head and Neck Squamous Cell Carcinomas
hnRNP: heterogeneous nuclear Ribonucleoproteins
HPV: Human Papilloma Virus
IHH: Indian HedgeHog
IPF: Idiopathic Pulmonary Fibrosis
IRES: Internal Ribosome Entry Site
ISE: Intronic Splicing Enhancer
ISS: Intronic Splicing Silencer
ITAF: Initiation of Translation Accessory Factor
LINE: Long Interspersed Nuclear Element
lncRNA: Long non-coding RNA
LTR: Long Terminal Repeat
NES: Nuclear Export Signal
NHEK: Normal Human Epidermal Keratinocytes
NLS: Nuclear Localisation Signal
NMD: Nonsense Mediated Decay
OD: Oligomerisation Domain
OSCC: Oral Squamous Cell Carcinoma
PABP: Poly(A) Binding Protein
PAF: Pol II Associated Factor
PAP: Poly(A) Polymerase
PAS: PolyAdenylation Signal

PTBP1/2: Polypyrimidine Tract Binding Protein $\frac{1}{2}$
p53/63/73: protein notation of the p53 family members
RBP: RNA Binding Protein
RPs: Ratcheting Points
RRM: RNA Recognition Motif
SAM: Sterile Alpha Motif
shRNA: short hairpin RNA
SINE: Short Interspersed Nuclear Element
siRNA: short interfering RNA
SMA: Spinal Muscular Atrophy
snRNP: small nuclear Ribonucleoprotein
SR: Serine/Arginine Rich domain containing proteins
SSO: Sequence Specific Oligonucleotide
TGF- β : Tumour Growth Factor β
TID: Terminal Inhibitory Domain
TNF: Tumour Necrosis Factor
TRAIL: Tumour necrosis factor-Related Apoptosis Inducing Ligand
TP53/63/73: gene or RNA notation for the *TP53* family members
UTR: Untranslated Region
WT: Wild Type
3'SS: 3' Splice Site
5'SS: 5' Splice Site

INTRODUCTION

A *TP63* centric view of the *TP53* family

I. The *TP53* family, an evolutionary toolbox for genome and tissue maintenance and integrity

For its continued prosperity, any species must be able to create healthy offspring. In order to do so, it is crucial that its germline be protected from stress-induced genomic instabilities. In modern day species, this protection can be found from humans all the way back to choanoflagellates and sea anemones, attesting of its early emergence and billion-year old importance. This function is carried by the *TP53* family genes. In humans, this ancestral gene family has three members: *TP53*, *TP63* and *TP73*. The earliest and unique member of this family of genes, as present in choanoflagellates and sea anemones, is closest to the modern *TP63/TP73* genes. Duplication of this gene led to the appearance of *TP53*. It is thought that *TP53* filled the need for a guardian of somatic tissue integrity as multicellular organisms grew more complex and began to rely on the proliferation of stem cells for adult tissue homeostasis. Recent analysis of the elephant shark genomic sequences identified all three family members in its genome. This suggested that two consecutive duplication events led to two *TP53* family genes in early vertebrate, rapidly followed by the appearance of a third member to compose the family as it is now known in all modern vertebrates. In all Gnathostomata, clearly distinct versions of *TP63* and *TP73* exist (Belyi et al. 2010; Lane et al. 2011). Recently published work on the family revealed that all three family members are already found in lamprey pushing back the origin of distinct *TP73* and *63* genes before Gnathostomata radiation about 500 Million years ago (Biscotti et al. 2019), see **Figure 1**.

All three proteins share a common basic organisation which reflects their role as transcription factors: an N-terminal transactivation domain, a central DNA binding domain, and additional C-terminal domains required for oligomerisation and regulation (**Figure 2**). The DNA binding domain of all three proteins is highly conserved but each family member diverges more significantly on the other functional domains. Globally *TP63* is closer to *TP73* than to *TP53*. Each family member has unique biological properties. The broad functions of these three genes in mammals have best been elucidated through a series of

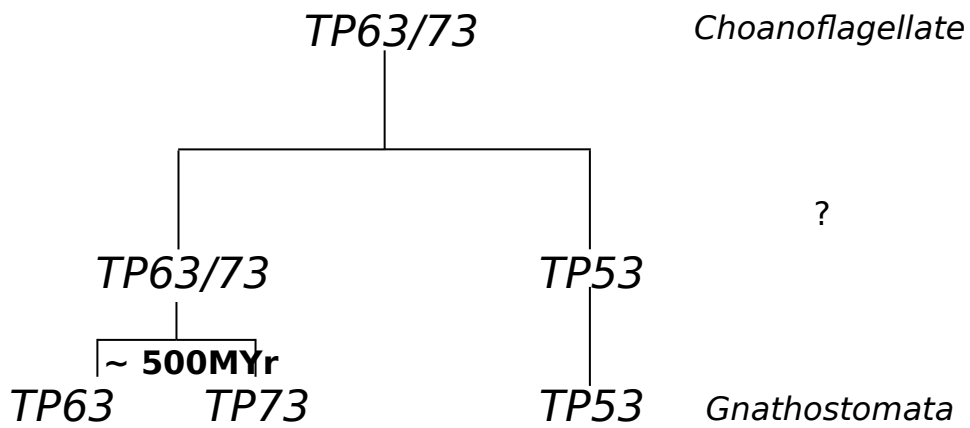


Figure.1. Schematic evolution of the TP63/73/53 gene family

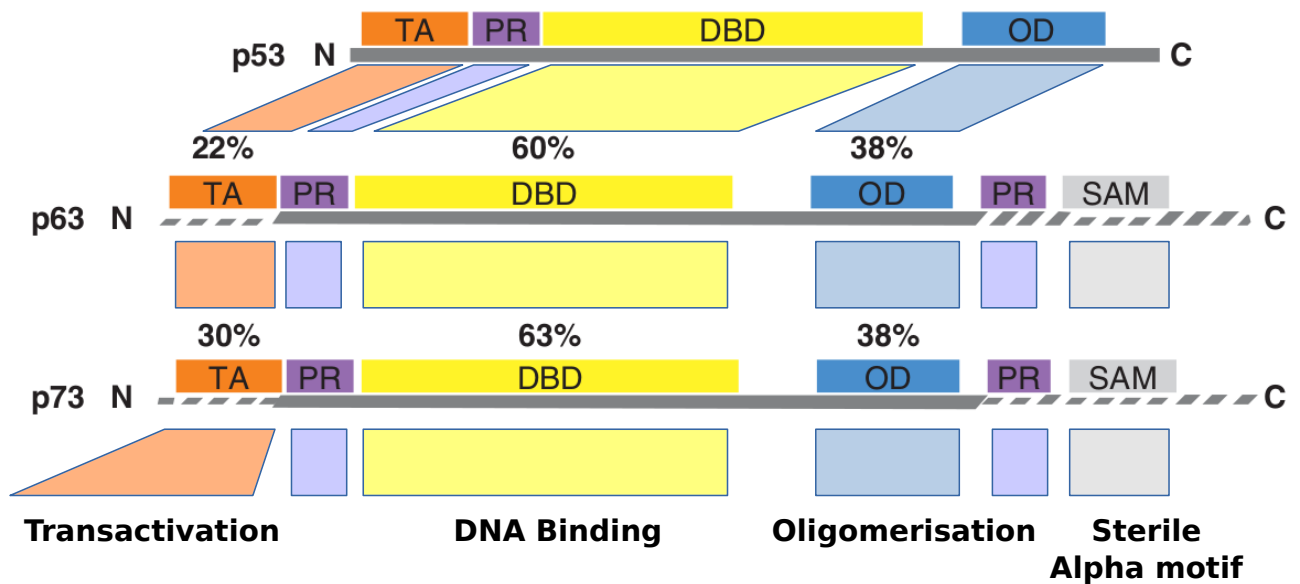


Figure. 2. Domain organisation and conservation of human p53/63/73. PR- proline rich regions. Conservation between p53/p63 and p63/p73 is shown for transactivation, DNA binding and oligomerisation domains. (Adapted from Dötsch et al. 2010)

knockout experiments in mice and studies of naturally occurring mutations in humans. Loss of *TP53* function in mice is associated with an increase in the early onset of multiple tumours (Lavigne et al. 1989; Donehower et al. 1992). Human mutations in *TP53* are causal to the Li-Fraumeni syndrome, a rare disease characterized by an autosomal dominant transmission and by a dramatic increase in many different types of cancers (Malkin et al. 1990).

TP73 knockouts are accompanied by dramatic developmental disorders: hippocampal dysgenesis, hydrocephalus, chronic airway infections and sterility but do not develop spontaneous tumours as opposed to *TP53* deficient mice (A. Yang et al. 2000). These effects are being mediated at least in part by *TP73*'s control of the differentiation of multiciliated cells (Napoli et Flores 2016). Mutations in the human *TP73* gene lead to reduced production of motile cilia that impact both respiratory and cognitive systems. Patients have defective respiratory functions, associated with respiratory infections because of impaired mucociliary clearance, and impaired brain development (Wallmeier et al. 2021).

TP63 was the last gene of the family to be cloned (A. Yang et al. 1998). *TP63* knockouts in mice lead to severe developmental defects in epidermis stratification, limb formation and fusion of craniofacial buds (Annie Yang et al. 1999). These data paint a clearer picture where *TP63* and *TP73* are crucial during differentiation and morphogenesis, whereas *TP53* acts as a somatic tumour suppressor. This is further demonstrated by the high frequency of *TP53* mutations in cancer, reaching around 50% (Baugh et al. 2018), whereas *TP63* and *TP73* mutations are rare in cancers. In Humans, heterozygous *TP63* mutations are the cause of several autosomal dominant syndromes frequently characterised by ectrodactyly, cleft lip and palate and ectodermal dysplasia, which I will develop on in a following dedicated chapter.

II. Three genes, many more isoforms

The structure and the regulation of the three genes is complex (**Figure 3**). The *TP53* family genes are generally under the control of two major alternative promoters producing mRNAs that differ in their 5' end. Combined with alternative splicing and sometimes alternative translation, this generates an impressive array of protein isoforms. The *TP53* gene's expression can lead to at least 12 different isoforms - p53, p53 β , p53 γ , Δ 133p53, Δ 133p53 β , Δ 133p53 γ , Δ 40p53, Δ 40p53 β , Δ 40p53 γ -(Anbarasan et Bourdon

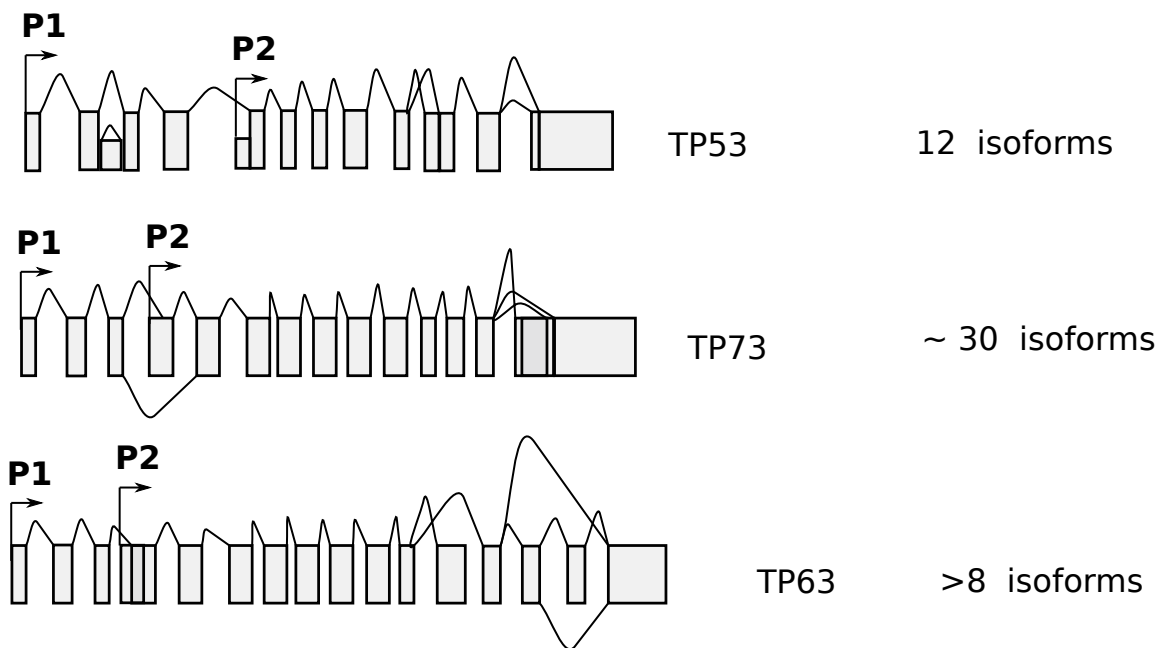


Fig. 3 : Alternative promoters and alternative splicing events produce many RNA and protein isoforms.
 Schematic representation of the complex exon/intron architecture of TP53/63/73 genes.

2019), the *TP63* gene's expression can lead to at least 8 different isoforms - TAp63 α , TAp63 β , TAp63 γ , TAp63 δ , Δ Np63 α , Δ Np63 β , Δ Np63 γ , Δ Np63 δ - and the *TP73* gene can encode in excess of 35 different RNA isoforms with a theoretical possibility of 29 different proteins (Murray-Zmijewski, Lane, et Bourdon 2006).

The p53 family proteins have a modular organisation with functional or regulatory domains sequentially distributed along the protein. I will take the structure of p63, the main focus of my thesis, as a paradigm (**Figure 4**). Three transcription initiation sites have been identified on the *TP63* locus (A. Yang et al. 1998; Beyer et al. 2011). They are termed P0, P1 and P2. These promoters generate 3 different 5' terminal exons (exon U1, 1 and 4). Four different translation initiation codons are described (AUG1, AUG2, AUG3, AUG4) and may produce 4 different N-termini that correspond to the isoforms GTAp63 (P0, ATG0), TA*p63 (P1, ATG1), TAp63 (P1, ATG2) and Δ Np63 (P2, ATG3). The 4 different encoded N terminal peptides are shown (**Figure 4A**) and are in the same reading frame. At the 3' end of the gene, two cassette exons (14 and 15) and 2 terminal exons (12 and 16) can produce multiple alternative C-terminal extremities. Because the alternative exons (15, 16) are not in the same reading frame, alternative splicing leads to frame shifting and a stop codon appears in the terminal exon (16) earlier than in the longest α isoform. Altogether this leads to the four different C-terminal peptides shown on the figure. Not only are the C- and N- terminal peptides different but full peptidic domains are omitted or diverged in-between the different isoforms. This is detailed in **Figure 4B-C**

The use of the proximal promoter (P1) leads to proteins which possess an N-terminal transactivation domain (TA) similar to the p53 transactivation domain, these are the "p53" isoforms and the "TAp63" or "TAp73" isoforms depending of the gene. The use of the distal promoters (P2) leads to proteins entirely or partially lacking a TA, these are the " Δ p53", " Δ Np63" or " Δ Np73" isoforms.

From N to C-terminal the longest TAp63 α isoform is composed of a transactivation domain, a DNA binding domain, an oligomerisation domain, a sterile alpha motif domain and a transcriptional inhibitory domain. Most of the alternative splicing events in the *TP53/63/73* pre-mRNAs take place closer to the 3' end, favouring the production of protein isoforms with variable C-terminal domains. It is striking that no alternative splicing events affect the tetramerization domain. The minimal domains common to all of the p63 isoforms are the DNA binding domain and the tetramerization domain. This minimal configuration corresponds to the Δ Np63 γ isoform.

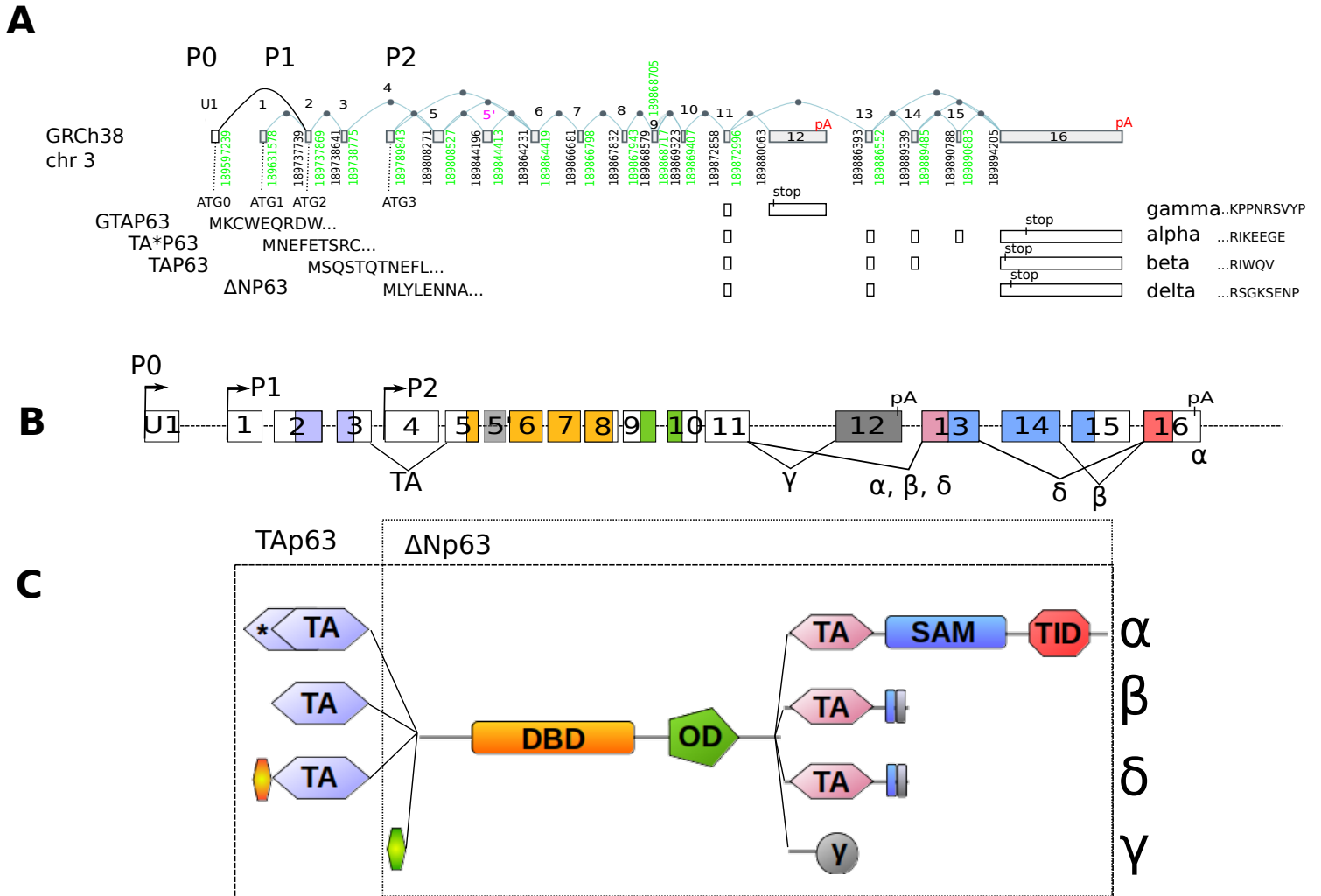


Figure 4 : TP63 gene, RNA and proteins.

A. Structure and genomic location of 5' and 3' splice sites of the *TP63* human gene. "pA" denotes polyadenylation sites. ATGn indicates the start codon. N and C-terminal peptides of the indicated isoforms are shown. Exon composition of the 4 main different C-terminal isoforms is shown under the gene structure. **B.** Exon structure and color encoded peptidic domain of TP63. **D.** Domain composition of the main P63 protein isoforms.

The active conformation of the p53 family transcription factors is a tetramer (a dimer of dimers, **Figure 5B**)(Brandt et al. 2009). The different p53/63/73 isoforms can bind to each other creating heterotetramers. The interaction is created via their oligomerisation domain. The homology between the oligomerisation domains of p63 and p73 enables them to bind to each other, however neither of them can bind to native p53 (Davison et al. 1999). Some mutated forms of p53 are however capable of interacting with p63 or p73 isoforms (S. Strano et al. 2000; Sabrina Strano et al. 2002; Bergamaschi et al. 2003; Gaiddon et al. 2001; Stindt et al. 2015). The formation of heterotetramers opens the door to many possible functional consequences on the recognition and the activation or repression of their target genes. Indeed, despite a conservation rate of around 60% between p53, p63 and p73 DNA binding domains (Dötsch et al. 2010) and highly similar DNA binding specificities (see **Figure 2 and 5**), their respective target genes appear to be mostly non overlapping, especially between p63 and p53 (Fontemaggi et al. 2002; Gallant-Behm et al. 2012; Riege et al. 2020).

III. Molecular functions and regulations of p63

The main focus of my thesis being the regulation and the functional consequences of the alternative splicing of *TP63*, I will therefore narrow down my field of study to the *TP63* transcription factor. We should however keep in mind that its interactions with the other family members may complexify the picture even more.

III.1. Different functions among different isoforms

III.1.1. *TAP63* vs Δ *NP63*

- differential expression

Based on GTEx data, there are 11 types of *TP63* positive tissues in a human body: skeletal muscle, bladder, testis, endocervix, ectocervix, mammary gland, prostate, minor salivary gland, vagina, esophageal mucosa and skin (See Results). Out of these tissues, *TATP63* is only expressed in testis, where it represents 96% of all *TP63*, and in skeletal muscle where it is the only N-terminal isoform detected (Marshall et al. 2021). *TATP63* is also expressed in the female germline where it participates in the maintenance of genome integrity. This concurs with evidence found in a prior study of human cell lines that concluded that Δ *NTP63* isoforms are mainly expressed in cell lines of mostly ectodermal origin, and *TAP63* is almost (with at least the exception of germ cells) only expressed in cell lines of mesodermal origin (Sethi et al. 2015).

- differential functions

At the protein level, the differentiating factor between TAp63 and Δ Np63 isoforms is that the former possess an N-terminal transactivation domain similar to that of p53, whereas the latter does not. This TA domain is functional as a p53-like transcriptional activity is observed following transfection of the different TA isoforms. The different Δ N isoforms are unable to transactivate a p53 reporter vector (Petitjean et al. 2008).

Δ Np63 isoforms are nonetheless not devoid of transactivation activity thanks to a second transactivation domain located between the oligomerisation domain (OD) and the sterile alpha motif (SAM) (see **Figure 2**). This big difference between the two classes of isoforms is enough to give them many diverging functions. TAp63 is mainly associated with apoptosis, cell cycle and muscle development, while Δ Np63 is associated with stemness, survival, senescence, oncogenesis and metabolism (Fisher, Balinth, et Mills 2020). These broad and diverging functions are a gross representation of the underlying interactions between p63 isoforms and the various cellular signalling pathways.

The current view is that TA isoform are mainly activated upon DNA damage to allow for a control of genome integrity while Δ Np63 isoforms control and interact with a vast array of downstream effector genes and signalling pathways that affect development and tissue homeostasis.

III.1.2. C-terminal isoforms

- differential expression

TP63 α is the major isoform, representing 67 to 97% of overall *TP63* in all tissues except skeletal muscle where *TP63 γ* is most abundant, around 85%. In contrast, in tissues besides skeletal muscle, *TP63 γ* only represents 0 to 24% of the expressed *TP63*. *TP63 β* falls somewhere between the two, representing 5-21% of all *TP63* in the epithelial tissues with highest *TP63* expression. These percentages represent RNA levels, and were obtained from the analysis of GTEx RNAseq data (Marshall et al. 2021). An RNAseq analysis of multiple human cell lines came to a similar conclusion in cells expressing Δ N*TP63*: *TP63 α* is the main isoform, several log folds higher than both *TP63 β* and *TP63 γ* . Again, *TP63 β* was found to be more expressed than *TP63 γ* , which was only detected at low levels in some head and neck squamous carcinoma cell lines (Sethi et al. 2015). One of the main caveats in the analysis of the expression of p63 C-terminal isoforms is the lack of protein data due to the absence of efficient and isoform-specific antibodies.

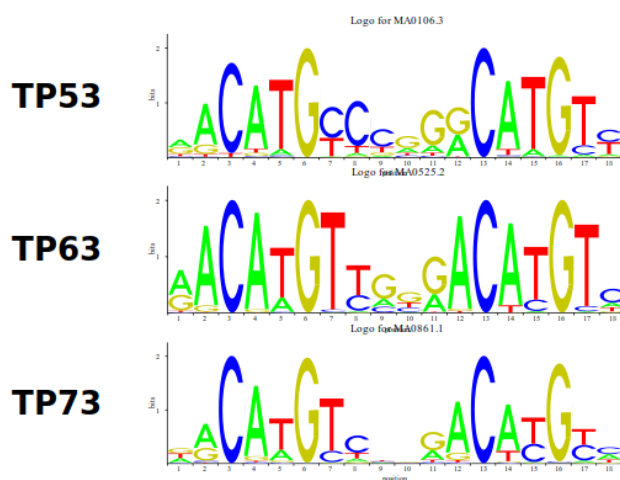
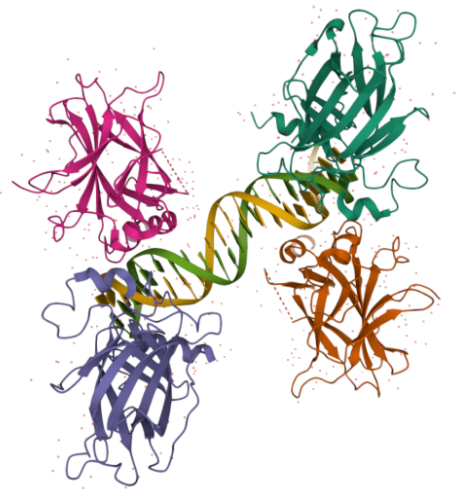
A**B**

Figure. 5. DNA Binding element and organisation of TP63 as tetramer. **A.** The DNA elements bound by the p63/73/53 proteins are presented as a logo motif based on the JASPAR 2016 database (<http://jaspar.genereg.net/>). **B.** Structure of the DNA binding domain of p63 associated with two response element (PDB : 3QYN)

- differential functions

The difference between C-terminal isoforms resides in the presence (or absence) of several functional protein domains. Yet, aside the TID that has a clearly demonstrated function in the repression of TA-domain dependent transcription upon DNA damage (Gebel et al. 2020), the SAM and the TA2 domain present in the α isoform are poorly described.

Δ Np63 α is the most expressed and the best described isoform. Δ Np63 α is involved in epigenomic and transcriptional control of epidermal differentiation. It is clear that the different C-terminal isoforms carry different functions as two different models of transgenic mice expressing only γ -like forms of p63 are unable to develop properly and present phenotypes highly similar but not identical to *TP63* knockout mice. This demonstrates that the α carboxy-terminal extremity is required for mice development and that the γ isoform is incapable of functionally substituting for it (Wolff et al. 2009; Suzuki et al. 2015). From a mechanistic point of view, the difference in the γ and α terminal regions is best defined in the context of the full length TAp63 isoforms. In this context, the transcriptional activity of p63 on p53-reporter genes is dependent upon the presence of the α or γ isoform. TAp63 γ is the most potent transactivator of a p53-dependent *CDKN1A* reporter gene. This is coherent with the *in vivo* results obtained in the mice described above as in this context, when only γ -isoforms were produced, the cell cycle was compromised due to *CDKN1A* activation (Suzuki et al. 2015). However it is yet not clear if some biological functions are specific to the γ isoform in the context of Δ Np63.

III.2. Regulation of *TP63* expression

As with many genes, different levels of regulation contribute to *TP63*'s ultimate expression level. These regulations can be transcriptional, post-transcriptional or post translational.

III.2.1. Transcriptional regulation of *TP63*

Many different pathways control *TP63* expression and dictate the usage of one promoter or the others. As stated previously, 4 different transcription initiation sites are reported for *TP63*, note P0, P1, P2. P0 is constituted by an endogenous retroviral long terminal repeat sequence and drives the expression of testis specific *GTATP63* (Pitzius et al. 2019). Little extra information is available as to its regulation. P1 historically drive the

expression of *TATP63* and *TA*TP63* respectively (A. Yang et al. 1998). It is however now thought that the isoform initially referred to as *TATP63* could be artefactual. The canonical *TATP63* isoform as we now know it, and as described as transcript variant 1 in the Uniprot database, is in fact the historical *TA*TP63* (Pokorná et al. 2021). P2 drives the expression of $\Delta NTP63$ (**Figure 4**).

The use of these different promoters must be tightly controlled in order to ensure the precise tissue specific expression of each class of isoforms. **Table 1** resumes the described transcription factors which are said to positively or negatively regulate *TP63* transcription. Of note, both p53 and p63 have been described as positive and negative regulators of $\Delta NTP63$. Knowledge of *TATP63* transcription regulators is far more limited, with only a handful of described regulators. Of all the described regulators, some act directly at the promoters, but several enhancer regions have also been described for the *TP63* gene. In the region corresponding to the first $\Delta NTP63$ exon there is an enhancer referred to as C38/C40 (Antonini et al. 2006; 2015a). When bound by $\Delta Np63$ itself or CEPB δ , it activates *TP63* transcription, whereas it is inhibited by CEPB α , CEPB β , or Pou3f1 (Leslie et al. 2017; Antonini et al. 2015a; Barbaro et al. 2007). An enhancer for the $\Delta NTP63$ promoter has also been described downstream of the *TP63* gene. In mice, SOX2 and PITX1 promote $\Delta NTP63$ expression via a distal enhancer (Sastre-Perona et al. 2019).

Chromatin modifications are also central in regulating *TP63* gene activity. The pioneer factor TFAP2C, which has been described for its role in preparing chromatin for epithelial lineage differentiation genes, binds to *TP63* in multiple locations and promotes its expression (L. Li et al. 2019).

Histone acetylation and methylation can influence $\Delta NTP63$ expression. Indeed, the histone acetyl-transferase inhibitor NOCL2 stimulates $\Delta NTP63$ expression in epidermal cells (Duteil et al. 2018). The histone demethylase KMD6A inhibits *TP63* expression by repressing one of its enhancers (Andricovich et al. 2018) whereas the histone methyltransferase DNMT3B promotes *TP63* expression by maintaining hydroxymethylation within its enhancers (Rinaldi et al. 2016). The switch between the TA and ΔN promoters is associated with a switch in their histone acetylation, which appears to be mutually exclusive. Analysis in cell lines has shown that in skeletal muscle myoblasts, histones surrounding the *TATP63* promoter show H3K27 acetylation while the $\Delta NTP63$ promoter showed the same marks in keratinocytes (Pokorná et al. 2021). Besides histone modifications, DNA methylation may also repress *TP63* expression as hypomethylation of the $\Delta NTP63$ promoter is associated with increased expression in lung squamous cell

Table 1: Transcription factors targeting the TP63 gene.
(Adapted from pokorna et al. 2021).

The last column indicates the affected isoform (orange Δ Np63, blue TAp63), the second column the impact of the promoter (green: upregulation, red: down regulation)

Transcription factor	Effect	Site	Validation	Promoter
p53	Up	-495 to -473	ChIP, mutation, mut-p53 OE	Δ Np63
p53	Up	Unkown	OE	Δ Np63
Mut-p53	Up			Δ Np63
Δ Np73	Up			Δ Np63
Sp1/Sp3	Up	-68 to -31	EMSA, mutation, ChIP	Δ Np63
NF-YB	Up	-126 to -96		Δ Np63
Oct6	Up	-152 to -121		Δ Np63
Δ Np63	Up	-164 to -61	OE, ChIP	Δ Np63
Δ Np63	Up	Unknown	OE	Δ Np63
Δ Np63	Up	-823 to -1	OE	Δ Np63
Δ Np63	Up	-404 to +139	OE	Δ Np63
Δ Np63	Up	-3043 to +139	ChIP, siRNA, mutation	Δ Np63
STAT3	Up	-557 to -539	ChIP, siRNA, mutation	Δ Np63
STAT3		-1.5kb	ChIP, siRNA	Δ Np63
STAT3		-719 to -693	ChIP, siRNA	Δ Np63
STAT3		-629 to -603	siRNA, mutation	Δ Np63
CTTNB	Up	-740 to +139	ChIP, siRNA	Δ Np63
CEBP δ	Up	-2.4kb	OE, siRNA, ChIP	Δ Np63
CEBP δ	Up	P2 and C38 C40	ChIP	Δ Np63
CEBP δ	Up	-433 to -142	ChIP, siRNA	Δ Np63
EGR1	Up			Δ Np63
Jun	Up			Δ Np63
Pax6	Up			Δ Np63
WT1	Up	-1110 to -960	ChIP, siRNA	Δ Np63
Ets1	Up	-2500 to -25	OE, mutation, ChIP	Δ Np63
FOXO3a	Up	-650 to -639	shRNA, OE, mutation	Δ Np63
GRHL2	Up	P2 sequence	OE, siRNA, ChIP	Δ Np63
SMAD3 SMAD5	Up	-350 to -1	ChIP, KO	Δ Np63
SMAD2	Up	-204	OE, mutation, ChIP	Δ Np63
DEC1	Up	-314 to +74	OE, siRNA, ChIP, mutation	Δ Np63
p65-RelA	Down	-841 to -716	ChIP	Δ Np63
δ EF1	Down	-2260 to -1830	KO, OE, ChIP	Δ Np63
Bhlha9	Down	-3000 to +1102	KO, OE	Δ Np63
TRPS1	Down	Two sites in P2	siRNA, ChIP	Δ Np63
Δ Np63	Down	-615 to -593	Mutation	Δ Np63
p53	Down	-495 to -473	OE	Δ Np63
p53	Down	-404 to +139	ChIP	Δ Np63
p53	Down	-3043 to +139	ChIP, siRNA, mutation	Δ Np63
GATA4	Down	-962		Δ Np63
CEPB β		-433 to -142		Δ Np63
B-catenin		-963 to -793	ChIP	TAp63
NFKB	Up	-650 to -636	mutation, EMSA, OE	TAp63
NFKB	Up	-538 to -528	mutation, EMSA, OE	TAp63
Sp1	Up	-86 to -77	mutation, ChIP	TAp63
FANCD2	Up	-801 to -321	ChIP	TAp63

carcinoma, whereas high methylation levels are found in normal tissue (Duteil et al. 2018; L. Li et al. 2019).

III.2.2. mRNA stability

The interaction between certain RNA binding proteins and TP63 mRNAs can have a drastic impact on their half lives, and subsequently on the downstream quantities of protein which can be produced. Rbm38 is capable of interacting with a AU/U rich sequence in the 3'UTR shared by *TP63 α* , *β* and *δ* (J. Zhang, Jun Cho, et Chen 2010). This interaction destabilises the *TP63* mRNA of these isoforms, but stabilises *TP63 γ* mRNA by interacting with its specific 3'UTR (Yan, Zhang, et Chen 2017). Rbm38 is induced during keratinocyte differentiation, placing it as one of the actors enabling cells to break away from the stemness-promoting effects of p63. Furthermore, Rbm38 is itself a target of p63, which creates a feedback loop dictating their relative abundance. The phosphorylation of Rbm38 at Ser-195 abrogates its effect on *TP63 $\alpha/\beta/\delta$* (Yanhong Zhang et al. 2019).

III.2.3. Protein stability and degradation

p63 isoforms are degraded in a proteasome-dependent manner after apposition of specific post-translational marks. Many enzymes have already been described for their participation in these modifications, mostly E3-ligases and kinases.

The first description of a p63-targeting E3-ligase came about in 2005 when it was described that the C-terminal region of p63 α contains target sequences for the E3-ligase Nedd4 and for the SUMO-conjugating enzyme Ubc9 (Bakkers et al. 2005). The p63 SAM domain present in α isoforms contains a PPPY motif which the E3-ligase Itch recognises and binds to. Itch can also recognise an 11 amino acid sequence in the N-terminus of all p63 isoforms, meaning Itch can target any p63 isoform to degradation (Rossi et al. 2006). The PPPY motif can also be recognised by WWP1, a homologue of Itch (Y. Li, Zhou, et Chen 2008). Some degradation pathways require the collaboration of multiple factors: MDM2 can bind to the SAM domain of p63 in the nucleus, provoking its translocation to the cytosol where the E3-ligase Fbw7 can then target it, in combination with the GSK3 kinase. This process cannot degrade p63 γ since it does not have a SAM domain (Galli et al. 2010).

In terms of kinases, I have already mentioned the regulation of TAP63 by c-Abl. This same kinase is reputed to be able to phosphorylate Δ NP63 α , albeit at different residues:

Y55, Y137 and Y308 (Yuan et al. 2010). Strikingly, the consequences are opposite, these modifications actually stabilise Δ NP63 α . The Δ NP63 α can however also be degraded following phosphorylation. Indeed, phosphorylation of residues S385, T397 and S466 mediated respectively by ATM, CDK2 and p70s6K all lead to its degradation (Huang et al. 2008). HIPK2 can also phosphorylate Δ NP63 α at the T397 residue (Lazzari et al. 2011). This illustrates the general importance of the C-terminal end of p63 for its regulation. Therefore the alternatively spliced isoforms of p63 may be differentially susceptible to those regulations which does not simplify the analysis of the functional consequences of these regulations.

The importance of the correct regulation of p63 stability was highlighted recently by the identification p63 as a major player in thalidomide teratogenicity. In the early sixties, many expectant mothers were prescribed thalidomide to help alleviate morning sickness. The steady flow of severe congenital birth defects that followed quickly led to its withdrawal from the market. The precise mechanism by which thalidomide use led to these defects remained a mystery for several decades. Some of the thalidomide-linked birth defects were strikingly similar to those which are observed in p63-linked human syndromes and prompted further enquiry. Using HaCaT cells and zebrafish embryos, a mechanism linking thalidomide, p63 and the birth defects was identified. Thalidomide treatment increases the activity of the CRL4^{CRBN} ubiquitin-ligase complex, which in turn targets both TAp63 and Δ Np63 for degradation. The degradation of TAp63 and Δ Np63 during embryogenesis respectively leads to developmental defects in the ear and in the limbs, explaining the deformities observed in newborns (Asatsuma-Okumura et al. 2019).

III.2.4. miRNAs targetting TP63

Micro RNAs (miRNAs), are short RNAs of around 22 nucleotides produced from intronic or intergenic regions which are capable of influencing gene expression by hindering translation and stability of their target RNAs. Their action relies on the base pairing of a “seed sequence”, around 6-8 nucleotides long, to their targets, generally within the untranslated regions. Because of the short seed-sequence, any given miRNA can potentially control hundreds of different mRNA targets. *TP63* isoforms can be regulated by miRNAs and are separated into two target groups based on the two different 3'UTRs which they can possess. On one side, *TP63 α* , *β* and *δ* , on the other, *TP63 γ* .

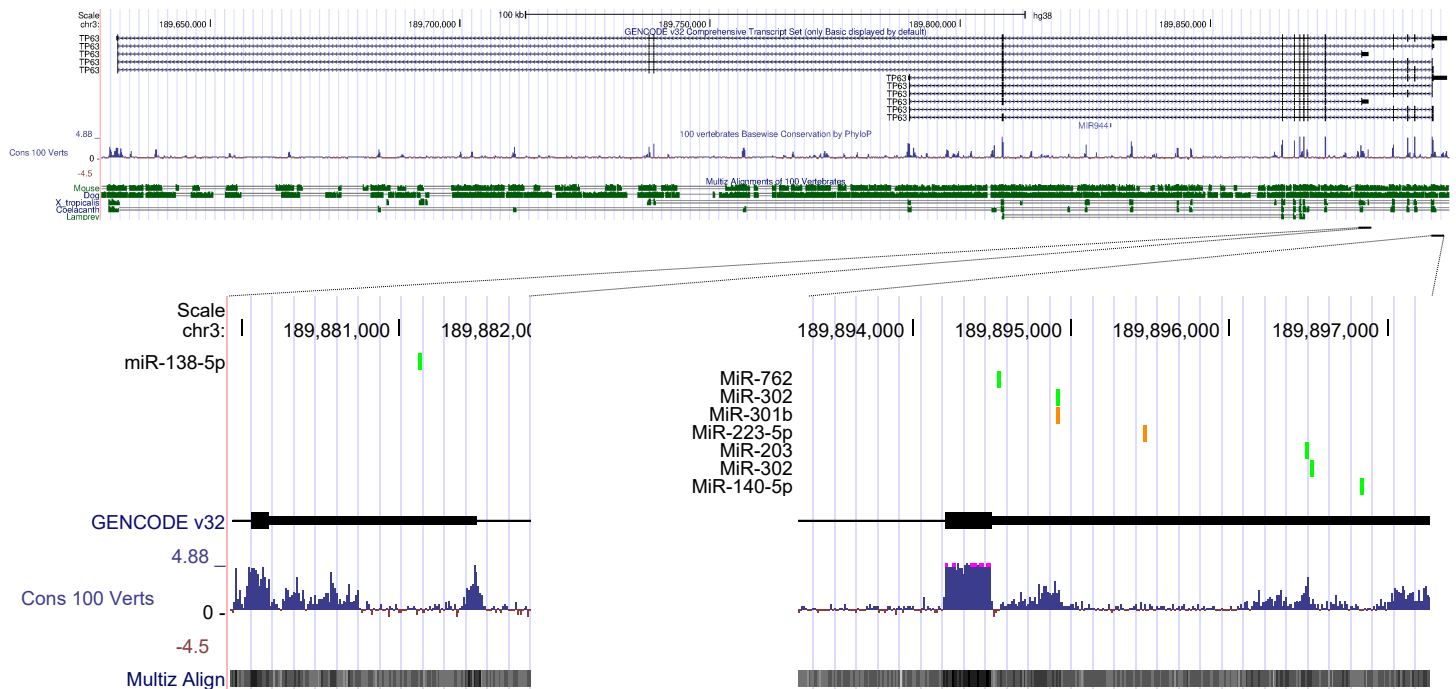


Figure. 6b. Location of binding sites for miRNA on the TP63 3'UTRs

Upper panel, genomic view of the Human *TP63* locus and mRNA isoforms. Lower panels, location of potential miRNA binding sites on the 3'UTR of the gamma (left) and alpha beta delta (right) *TP63* isoforms.

Studies of mouse keratinocyte differentiation *in vitro* have shown that as cells terminally differentiate, with increased levels of involucrin, there is an inverse relation between p63 and mir203 levels, mir203 levels increasing and p63 levels decreasing. In mice, transgenic overexpression of miR-203a-3p in the basal epithelial layer using a keratin 14 promoter construct (Yi et al. 2008) led to a decreased proliferation and an early activation of the epithelial differentiation program with accumulation of Keratin 10 expressing cells and depletion of proliferating basal cells. These changes were accompanied by downregulation of *TP63* expression. The up-regulation of miR-203a-3p in epithelial differentiating cells could be recapitulated *in vitro* using CaCl₂ induced differentiation of HaCaT cells. In this context differentiation induced upregulation of mir203 and downregulation of *TP63* mRNA. Specifically blocking miR-203 using an anti-miR oligonucleotide abolished the downregulation of *TP63* mRNA prompting to hypothesize a direct role of miR203 on the control of *TP63* mRNA. Bioinformatic analysis identified two binding sites for miR203 in the 3'most terminal exon of *TP63* at two positions of the human and mouse 3'UTR shared by $\Delta NTP63\alpha$, β and δ (Lena et al. 2008; Yi et al. 2008). A direct regulation of miR-203 on the level of *TP63* mRNA was confirmed using reporter assays and mutations of the miR-203 binding sites (Lena et al. 2008). Together, all these data make the case for a regulatory role of mir-203 in epidermal differentiation via downregulation of *TP63*. Yet, the regulation of *TP63* by this miRNA may be somehow tissue specific as in a bladder carcinoma cell line that robustly expresses p63, no impact of miR-203a-3p on *TP63* expression could be observed in the course of a screen for miRNA controlling TP63.

The importance of miRNA modulation of TP63 expression is further revealed in the case of development of Idiopathic pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) is a type of interstitial pneumonia which was triggered by neither infection nor cancer. In this disease, affected areas of the lung show an increase in both TAp63 and $\Delta Np63$ isoforms. In BEAS-2B and A549 cells, *TP63* expression is required for TGF- β 1 induced fibrosis, which is worsened by *TP63* overexpression. During IPF, miR184 is robustly downregulated according to multiple publicly available datasets (GEO accession numbers: GSE13316, GSE32638, GSE27430, GSE24323), as well as in tested tissue samples when compared to healthy tissue. In the BEAS-2B and A549 cells, overexpression of mir184 inhibited TGF- β 1-induced fibrosis, which could be partially reversed by concomitant *TP63* overexpression. In mice treated with bleomycin (BLM) to develop IPF, p63 protein levels are increased and mir-184 levels are decreased. The experimental overexpression of mir-

184 reduces the p63 increase. *TP63* therefore seems convincingly involved in the development of IPF, secondary to outside stimuli (J. Li et al. 2021). Which *TP63* isoforms are involved in this disease has not been investigated.

IV. p63 developmental functions

Many different *TP63* mice models have been developed in order to address different aspects of p63 developmental functions (Vanbokhoven et al. 2011). Studying their developmental defects was the starting point in understanding the biological importance of *TP63*. Initially, the lack of skin and limbs were the most obvious consequences of *TP63* knockouts, but p63's developmental importance has now been broadened to most epithelial tissues, and some non epithelial tissues such as bone and muscle.

IV.1. p63 in the establishment of the epidermis

Protection against the harshness of the outside environment is a vital need for all living organisms. In many animals, this protection is provided by the largest organ of the body, the epidermis, and its many different types of specialisations. Depending on the environment and the stage of development, epidermis can be as varied as hairy skin with sebaceous glands, feather-displaying skin, or muco-ciliary epithelia.

Briefly after cloning of the *TP63* cDNA, knockout experiments of *TP63* in mice (Nota: *TP63* is also called *Trp63* in mice) showed a severe lack of epidermal stratification leading to desiccation and death of the newborn pups (Annie Yang et al. 1999). These first experiments underlined the essential role of *TP63* in generating an epidermis. Immunohistochemistry of the epidermis of perinatal pups revealed a strong expression of p63 localised in the basal layers of the epidermis. Analysis of the molecular markers of the simple epithelium present at E12 in both control and knockout mice, before any stratification happens, revealed that the knockout mice lacked keratins 5 and 14 compared to the control mice. These markers are normally expressed by the basal cells. At E17 the control mice embryos have a stratified epidermis while knockout mice only present dispersed clusters of cells. In both cases, differentiation markers loricrin, filaggrin and involucrin are expressed. These data indicate that in the absence of *TP63*, there is a lack of basal progenitor cells, a lack of proliferation and terminal differentiation happens with no stratification.

Fluorescent labelling of cells of the stratifying epidermis in post-larval zebra fish embryos has shed some light on the chronological importance of p63 in its establishment (Rangel-Huerta, Guzman, et Maldonado 2021). Different populations of cells compose the stratified epidermis of the late zebrafish larvae. Epidermal Stem cells located closer to the basal membrane proliferate moderately and generate committed progenitor cells through asymmetric cell division. These epidermal stem cells express the highest levels of p63. The committed progenitor cells are highly proliferative cells of the basal and suprabasal compartments and generate the cells that make up the upper layers of the epidermis. They express low levels of p63 and one of their hallmarks is the increased phosphorylation of ser66/68 of p63. This phosphorylation event is involved in the cytoplasmic relocalisation of p63 and increased degradation. As cells move upwards in the suprabasal compartment, p63 expression is completely lost. Furthermore, the rise in proliferative capacity is correlated to a rise in the levels of phosphorylated p63, which is mostly located in the cytoplasm. These findings are close to what has been described in human epidermis (Suzuki et Senoo 2012) as well as during wound healing of adult mouse skin (Suzuki et Senoo 2013) where p63 was detected both in basal and suprabasal layers, with an increase in phosphorylated p63 in the suprabasal layer as p63 levels decrease.

In vitro, Ca²⁺ induced differentiation of human keratinocytes leads to this same increase of phosphorylated p63 and decrease of total p63. In long term cultures of epidermal holoclones (cultures derived from the epithelial stem cells), the cells showing higher levels of p63 phosphorylation have a clearly lower proliferative capacity (Suzuki et Senoo 2012). Together, all these data point in the same direction: p63 is required for the establishment and maintenance of a population of basal stem cells. Loss of p63, which can be secondary to its phosphorylation, guides cells towards the transiently amplifying stage, and its ultimate loss allows cells to terminally differentiate after a few rounds of divisions. Phosphorylated p63 can therefore be used as a marker of the initial stages of epidermal differentiation.

This however raises the question of the initial activation of TP63 expression in the basal cells of the epidermis. Work realised in *Xenopus laevis* embryos provided some clues about the triggers of TP63 expression. The epidermis of the *Xenopus* larvae proved to be an excellent model of muco-ciliary epithelium development (Walentek 2021). As shown on **Figure 6**, early TP63 expression is first detected in the non neural ectoderm and delineates the neural ectoderm as neurulation progresses (Yann Audic, unpublished data). Haas and colleagues (Haas et al. 2019) identified the Wnt/ β -catenin signalling pathway as

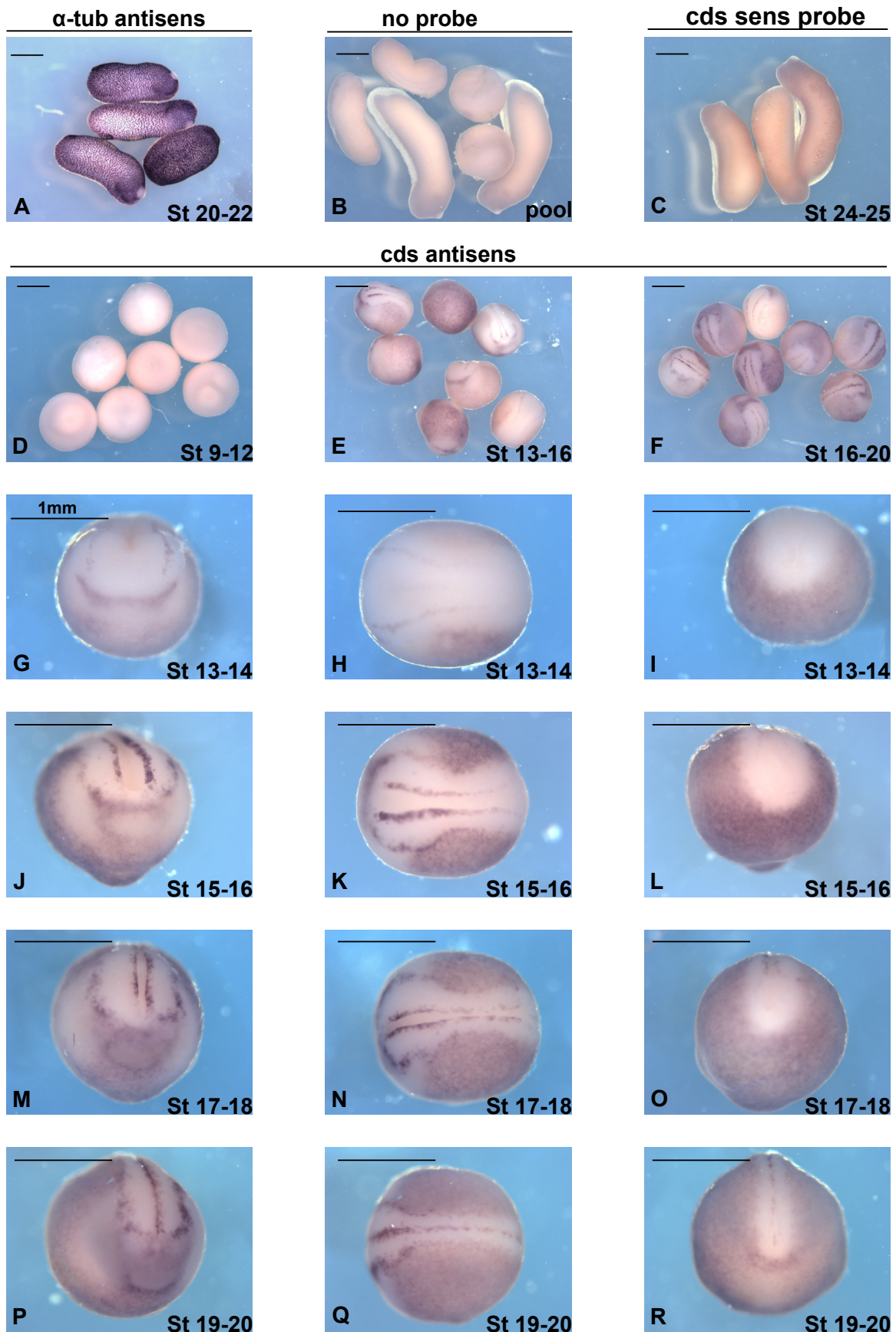


Figure. 6. TP63 mRNA expression in developing *Xenopus laevis* by whole mount in situ hybridization.

(A) positive and (B-C) negative control probe for ISH. (D-F) pools of embryos at the indicated stage were used to detect *TP63* expression. (G, J, M, P) Anterior view of *TP63* expression in embryos at the indicated stages, (HKNQ) dorsal views, (I,L,O,R) posterior views. The scale bar is 0.5 mm. (from Yann Audic, unpublished data)

a major regulator of *TP63* expression in the developing epidermis, in line with previous work that demonstrated the direct binding of β -catenin on the Human and *Xenopus TP63* promoter (Ruptier et al. 2011; Kjolby et Harland 2017). The induced expression of $\Delta NTP63$ led to increased proliferation and reduced differentiation of the multi-ciliary cells showing that p63 controls the proliferative potential of basal cells of the *Xenopus* epidermis.

IV.2. p63 regulates many other epithelia

Epithelia are not limited to skin and on the contrary are among the earliest evolved tissues in multicellular organisation. They can derive from all three embryonic layers. *TP63* expression can be found in all types of epithelia from ectodermal, endodermal, or even mesodermal origin and delineates epithelial stem cells in these different contexts. A nice example (**see Figure 7**) from this epithelia-wide *TP63* expression could be found in the early work of Annie Yang on *TP63* cloning (A. Yang et al. 1998) where clear p63 labelling could mostly be observed in the basal cells of epithelia from ectodermal, mesodermal or endodermal origin.

A series of interesting xenograft experiments of rat epithelial stem cells onto mice have shown $\Delta NTP63$ expression to be associated with a potential for multipotency. Indeed, $\Delta NTP63$ expressing cells from squamous epithelia can be xenografted and lead to successful establishment of hairy epidermis with development of hair follicles and sebaceous glands. $\Delta NTP63$ expressing cells from epithelia of different embryonic origins can also give rise to hair epidermis and sebaceous glands following an intermediary culture step, with the exception of tracheal cells which never successfully produce sebaceous glands or hair follicles. Interestingly, despite differentiating into hair epidermis, the grafted cells kept expressing transcription factors associated with their tissue of origin (Claudinot et al. 2020). This work therefore highlights the pluripotency of $\Delta NTP63$, which rather than allowing for trans-differentiation, seems to keep cells open to commit to a multitude of fates in a reversible manner. This is in line with the discovery that $\Delta NTP63$ expression is a favourable factor in the reprogramming of differentiated cells using some of the Yamanaka transcription factors (Alexandrova et al. 2013). The ultimate fate of the differentiating cells being determined by the cellular environment and external clues in which it is developing. This suggests that p63 expression patterns are probably quite similar in all stratified epithelia, with expression in stem cells and loss towards terminal differentiation.

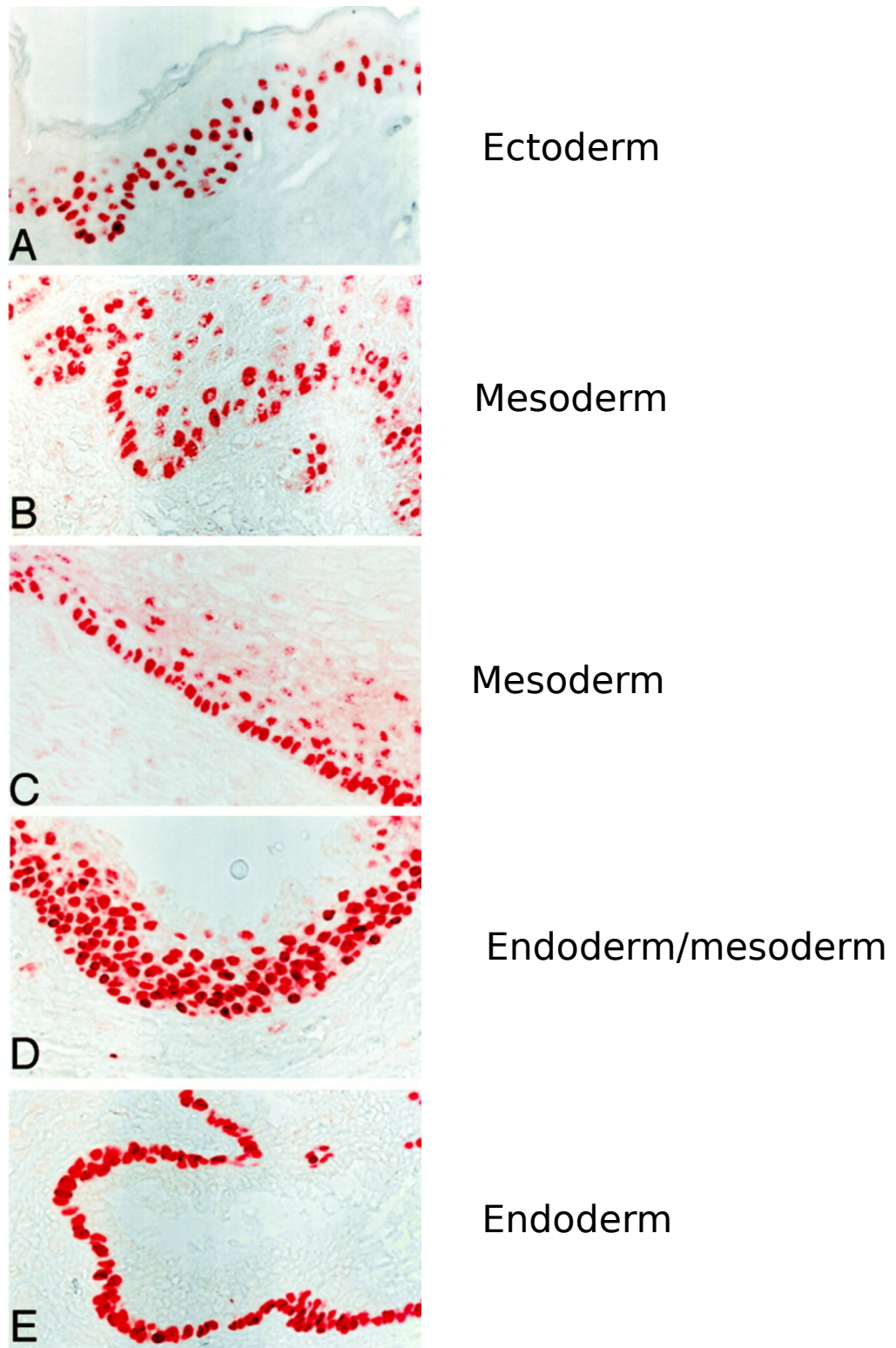


Figure 7. Immunolocalization of p63 in Human Epithelial Tissues from Yang et al. 1998

Paraffin sections of normal human epithelial tissues probed with monoclonal antibodies to p63 using an alkaline phosphatase reporter system. **A.** p63 staining in foreskin showing nuclear localisation of p63 in basal epithelial cells. **B.** p63 localisation to basal cells of ectocervical epithelium. **C.** p63 localisation in basal cells of vaginal epithelium. **D.** p63 staining of basal urothelium cells. **E.** p63 staining of the epithelial cell layer below luminal cells in prostate tissue.

The striking epidermal defects and the early lethality associated with *TP63* knockout initially masked the many diverse developmental functions of *TP63*. From the perspective of what we now know of *TP63*, it is striking that “almost” normal embryos could develop from animals defective in *TP63* expression. One could have envisaged that early defects in *TP63* could lead to much more drastic phenotypes.

IV.2.1. p63 in the eye: corneal regeneration

In mammals, the integrity of the cornea is ensured by a stock of corneal epithelial stem cells. These stem cells are preferentially located in the limbus, a ring-like structure that surrounds the cornea and delimits it from the surrounding sclera. Similarly to what has been described in the epidermis, in the ocular surface epithelia, $\Delta NP63\alpha$ is the most dominant isoform, with lesser expression of $\Delta NP63\beta$ and $\Delta NP63\gamma$, as determined by isoform specific RT-PCR. No TA isoforms were detected in the eye (Kawasaki et al. 2006). Immunofluorescence staining of p63 in normal human cornea indicates it is present in basal limbal cells but nearly absent from the cornea. $\Delta Np63$ localizes both to the limbal stem cells and to the committed central cells of the cornea, $\Delta Np63$ expression is lost in the most differentiated superficial cells (Robertson, Ho, et Cavanagh 2008). Following corneal injury however, p63 is detected in the basal and suprabasal layers of both the limbus and the cornea. Isoform specific *in situ* hybridization analysis of the *TP63* mRNA expressed in these tissues showed that in the injured corneas there is a significant increase in $\Delta NTP63\beta$ and $\Delta NTP63\gamma$ in the suprabasal layers, in opposition to the $\Delta NTP63\alpha$ mRNA which was strictly confined to the basal layer. The p63 positive cells found in the cornea following injury are cells that have migrated from the limbal tissue to regenerate the cornea (Di Iorio et al. 2005). It is not clear at this stage what role the shift in p63 isoforms from basal to suprabasal compartments plays in the physiological process of corneal wound healing. In the clinical context, p63 positivity is used as a positive predictor of success of regenerative therapies which relies on grafting of limbal holoclones to eyes which have suffered corneal loss or deterioration (Rama et al. 2010).

IV.2.2. p63 in the uterus and vagina

Both the uterus and the vagina derive from the Müllerian ducts. Once mature, the uterus is lined by a simple columnar epithelium, while the vagina is lined by stratified squamous epithelia. The uterus and the proximal 1/3 part of the vagina are of mesodermal

origin while the 2/3 distal part is of ectodermal origin. The basal cells of the vagina express $\Delta Np63\alpha$, while those of the uterus do not (Takeshi Kurita et al. 2005). This is therefore an interesting model to study how the switch between commitment to stratified epithelium or simple epithelium is operated. The Müllerian ducts from *TP63* *-/-* mice pups show normal uterine development, however their vagina lacks a stratified epithelium. Recombination grafting experiments where neonatal uterine mesenchyme is associated with vaginal epithelium fail to allow vaginal p63 expression and epithelial stratification. Conversely, when vaginal mesenchyme is associated to neonatal uterine tissue, epithelial uterine expression of p63 and subsequent stratification is triggered (T. Kurita et Cunha 2001). Therefore it is likely that clues from the neighbouring tissues are necessary to instruct cells to express *TP63* and favour epithelial stratification. In the context of female genital tract development, the expression of *TP63* was shown to be under the control of the estrogenomimetic diethylstilbestrol (DES). Exposure of mice fetuses to diethylstilbestrol (DES) provokes cervico-vaginal adenosis, which is characterized by the presence of simple columnar epithelium instead of a stratified columnar epithelium. At least one aspect of this disease is based on the loss of p63 expression following DES exposure (Takeshi Kurita, Mills, et Cunha 2004). This places $\Delta NP63\alpha$ as a major trigger for epithelium commitment towards the stratified squamous type, and acts in response to mesenchymal clues which have yet to be identified in this context.

IV.2.3. p63 in mammary tissue development and maintenance

TP63 *-/-* mice as well as $\Delta NTP63$ specific knockout mice are devoid of mammary glands (Annie Yang et al. 1999; Mills et al. 1999; Chakrabarti et al. 2014; Koster et al. 2007), indicating that one or several of the $\Delta Np63$ isoforms are required for their development. When reintroduced into cleared mammary fatpads, epithelial stem cells expressing $\Delta NTP63$ from lentiviral vectors are capable of partially regenerating the missing organ, contrary to epithelial stem cells expressing *TATP63* (Chakrabarti et al. 2014). Interestingly, the stem cell capacity of the $\Delta NTP63$ transduced epithelial stem cells was critically dependant upon Wnt signalling and the expression of WNT5A and FZD7, a receptor and a ligand of the Wnt pathway, was upregulated upon overexpression of $\Delta NTP63$ in a human mammary epithelial cell line. It is now thought that at the molecular level, $\Delta Np63$ promotes stemness through activation of the Wnt/ β -catenin pathway (Lindley et al. 2015). The stemness properties induced by the p63 expression are often associated

with dedifferentiation as can be observed when loss of p63 in mammary basal cells shifts their expression of markers away from the basal and towards the more luminal phenotype, and oppositely when ectopic expression of p63 in luminal cells shifts them back towards a more basal phenotype (Yalcin-Ozuysal et al. 2010).

Epithelia are dynamic structures whose integrity is kept balanced by a moderate renewal of the cells composing the epithelium and a progressive shedding of the oldest differentiated cells. Stem cells are generally not highly proliferating cells but rather slow dividing cells that generate and eventually replenish (in the case of injury) a population of transient amplifying cells or progenitor cells with higher proliferative capacities. These transient amplifying cells then differentiate.

While most of the basal cells of epithelia express high levels of $\Delta Np63$, the balance between TAp63 and $\Delta Np63$ seems to sometimes play a regulatory role in balancing stemness with proliferation. Using a complex cell sorting procedure, Li and colleagues identified mammary cell populations that expressed either TA or $\Delta Np63$. The enriched stem cell fraction express $\Delta Np63$ in agreement with previous evaluation of $\Delta Np63$ expression in stem cell while a progenitor cell population exclusively expressed TAp63 along with the Indian hedgehog (*IHH*) ligand. The strict exclusion of *IHH* expression and $\Delta NTP63$ expression raised the possibilities that *IHH* is controlled by p63 or controls *TP63* promoter selection. Overexpression of the TAp63 γ isoform or downregulation of $\Delta Np63\alpha$ activated *IHH* expression in immortalized mammary epithelial cells, demonstrating that TAp63 enhances hedgehog signalling. Both TA and $\Delta Np63$ associate to the *IHH* locus in ChIPseq experiments. Furthermore, *IHH* activation depleted the hedgehog effectors GLI3/GLI3R, increased mitotic activity in the progenitor population and favoured TAp63 expression rather than $\Delta Np63$, as did GLI3 down-regulation. Altogether, this indicated that in mammary glands, a reciprocal interaction between p63 and *IHH* controls the production of progenitor cells from Mammary stem cells (Na Li et al. 2008). Interestingly, inhibition of Hedgehog signalling pathways by deletion of the *SMO* gene (Smoothened) in ectodermal tissues by conditional knockout led to the production of a glandular epithelium somehow capable of differentiation into mammary glands (Gritli-Linde et al. 2007). This suggests that control of proliferation and differentiation in a p63 context is highly dependent of the hedgehog signalling pathway.

IV.3. p63 in non-epithelial tissue developmental

IV.3.1. Limbs/bones

Besides the lack of any stratified squamous epithelium, *Trp63*^{-/-} mice also showed severe limb and craniofacial development issues. p63 is normally found in the apical ectodermal ridge (AER) of the limb buds. At the RNA level, $\Delta NTP63\gamma$ is the main isoform expressed during limb development from E10.5 to E12 in normal mice. $\Delta NTP63\alpha$ but not $\Delta NTP63\beta$ is also expressed during this period, at lesser levels than $\Delta NTP63\gamma$ and there is a progressive increase in *TATP63* expression. *Fgfr8* and *Fgfr4*, positive regulators of the AER, are proposed transcriptional targets of $\Delta Np63\gamma$. *Jag2*, a negative regulator of the AER, seems to be a target of *TAp63\gamma* (Kawata et al. 2017a).

Study of mouse chondrocytes *in vitro* has shown that the shift from proliferative cells, which express mostly *TATP63* α and β , to hypertrophic cells is accompanied by a significant increase in *TATP63\gamma* and $\Delta NTP63\beta$ mRNA (Gu et al. 2013a). Depletion of $\Delta NTP63\alpha$ in mouse chondrocytes has no effect on the ossification process of limbs, digits and tail bone. Depletion of *TATP63\alpha* however provokes a delay in the ossification of digits and tailbone in mice (Lu et al. 2013). Overexpression of *TATP63\alpha* in mouse chondrocytes *in vivo* leads to accelerated ossification. *In vitro*, overexpression of *TATP63\gamma* in ATDC5 cells, a mouse cell line used for *in vitro* studying of skeletal development (Yao et Wang 2013a), led to an increase in *col10a*, a marker of hypertrophic differentiation.

The exact participation of different P63 isoforms in the formation and limbs and the ossification process that ensues is therefore unclear. There is an apparent importance of p63 γ in the establishment of limbs as opposed to the dominant function of p63 α in the establishment of epithelia.

IV.3.2. Muscle

shRNA mediated knockdown of *TATP63* in spontaneously immortalised C2C7 mouse muscle cells increases their proliferation rate by removing CDKN1A and CDKN1C inhibition. Overexpression of *TATP63\gamma* in this same cell line led to a decrease in proliferation (Ciuffoli et al. 2018). *In vitro* differentiation of C2C7 cells leads to a vast increase of *TATP63\gamma*, while knockdown of *TATP63* in these cells impairs their capacity to organise into myotubes (Cefalù et al. 2015). Immunohistochemical staining of tissue samples from various types of striated skeletal derived cancers has shown that p63 has a cytoplasmic expression in these cells. Furthermore, immunoelectron microscopy identified

p63 presence at the Z striations. This is one of the rare indications of a non-transcription related function of p63, which could therefore be involved in myofibril organisation or function (Martin et al. 2011).

IV.3.3. Heart

The severity of the epidermal phenotype of *TP63* *-/-* mice is such that it may have led to other potentially lethal phenotypes being overlooked. In work published in 2011, the impact of *TP63* deficiency on cardiac development was identified (Rouleau et al. 2011). Without *TP63*, mouse pup hearts present with ventricular dilation, thin ventricular walls, poor trabeculation and impaired myofibrillogenesis. Besides this, the mitochondria also seemed to be affected, impaired mitochondrial function in muscle cells has since also been identified (Ciuffoli et al. 2018). The defects in heart development could explain in utero mouse mortality, as well as participate to the already well established peri-natal mortality of the pups. Strikingly though, p63 is not expressed in the E18.5 fully developed heart, or in any of the cells of the heart lineage in previous stages. Instead, p63 seems to be expressed by the associated endoderm. During the differentiation of mouse embryonic stem cells into embryonic bodies with beating cardiomyocytes, there is a switch in expression of *TP63* mRNA. *TATP63* is expressed during the first stages, and progressively Δ *NTP63* isoforms become the most abundant. Depleting *TP63* in these cells by shRNA leads to embryonic bodies devoid of beating cardiomyocytes. This phenotype is rescued by the transfection of GATA6 expression plasmids. GATA6 is a factor known to be involved in both endodermal formation and cardiogenesis. Furthermore, ChIP-seq analysis revealed activin-a as a target gene of p63. Activin-a is an endodermal stimulator of early cardiogenesis. Similarly as the expression of GATA6, addition of exogenous Activin-a to cultures of sh-*TP63* embryonic stem cells restored their ability to form beating cardiomyocytes. Together, these data (Rouleau et al. 2011) show that *TP63* expression in the embryonic endoderm participates in its crosstalk with adjacent mesoderm, stimulating some aspects of cardiogenesis. Analysis of the *TP63* isoforms in the END2 endodermal cell line revealed *TATP63* γ to be the dominant isoform. It is not clear at this point whether this is also the case in vivo, which would be interesting to confirm a non-epithelial role of the γ isoform.

IV.3.4. Cochlea

Some patients with *TP63* mutation-related ectodermal dysplasia suffer from hearing loss. Analysis of the E18.5 *TP63* *-/-* mouse embryos showed defects in the cochlear duct. RT-PCR analysis of *TP63* isoforms expressed in the cochlea of wild type E18.5 mouse embryos revealed that *TATP63* is present rather than $\Delta NTP63$. Furthermore, *TATP63* *-/-* mice show variable levels of sensorineural deafness. Microarray analysis of gene expression in SaOs-2 cells (human osteosarcoma) revealed that TAp63 α regulates several members of the Notch pathway, including *Hes5* and *Atoh1* for which putative p63 response elements have been identified. Both genes are downregulated in E18.5 *TAP63* *-/-* cochlear extracts. These data show a clear development role for TAp63 α in the maturation of the cochlea (Terrinoni et al. 2013).

V. p63 in germline protection

So far, we have discussed the crucial role of $\Delta NP63$ in epithelial homeostasis. The oldest function of the entire p53 family, however, lies in protecting the integrity of the germline. This function is still ensured today by a p63 homologue in *Caenorhabditis elegans* (Schumacher et al. 2001; Derry, Putzke, et Rothman 2001), *Drosophila melanogaster* (Ollmann et al. 2000), *Ciona intestinalis* (Heering et al. 2016), and sea anemones (Belyi et al. 2010). In all these species, as well as in mammals, p63 or its orthologues are activated following DNA damage (Gebel et al. 2020). Just like the functions in stratified epithelia seem specific to $\Delta Np63$ isoforms, germline protection seems to be the speciality of the TA isoforms and relies on the activity of the first transactivation domain. Indeed, $\Delta NTP63$ knockout mice phenocopy the knockouts of all isoforms, and specific *TAP63* knockout mice are not accompanied by developmental defects, indicating that TAp63 isoforms are dispensable for $\Delta Np63$ -like developmental functions. However, TAp63 is strongly expressed in oocytes, and in *TAP63* *-/-* mice, primordial follicular oocytes survive exposure to UV radiation which is lethal to them in WT mice, indicating that TAp63 is involved in eliminating oocytes following DNA damage (Suh et al. 2006). The p53 status had no influence on oocyte survival, despite its role as a DNA damage sensor in somatic cells. The activation of TAp63 leads to activation of pro-apoptotic factors such as PUMA and NOXA (Kerr et al. 2012).

In the mammal female germline, oocytes go through an important step before meiotic division that ensures maximum genetic recombination. At the zygotene stage of

prophase I, the chromosomes are aligned and subjected to many double stranded breaks by the type II topoisomerase Spo11 in order to promote crossovers (Keeney, Giroux, et Kleckner 1997). Having had the chance to crossover, most of these breaks are repaired by homologous recombination enzymes DMC1 and RAD51 (Hinch et al. 2020). Five days after birth, mouse pup oocytes are strongly positive for TAp63 (Suh et al. 2006). By this point oocytes are arrested in prophase I of meiosis (dictyate arrest), and TAp63 may eliminate those with enduring DNA damage, ensuring only a healthy pool of oocytes remains. Following this, the oocytes will stay blocked until many years later before luteinising hormone increase jumpstarts their cell cycle to become mature for ovulation.

In oocytes which show no signs of DNA damage, TAp63 is maintained in an inactive dimer conformation. DNA damage triggers the phosphorylation of TAP63 by the tyrosine kinase c-Abl at residues 149, 171 and 289. This provokes the loss of interaction of the terminal inhibitory domain with the transactivation domain. The dimers can then regroup as active tetramers, with high affinity for DNA targets. The C-terminal region of Δ Np63 α appears crucial in the inactivation of TAp63 by interacting with and masking the TA domain in the dimer conformation (Gebel et al. 2020). The downside, for TAp63, is that a tetrameric conformation exposes the consensus sites for binding to Itch and MDM2, the E3-ligases that target it for degradation (Rossi et al. 2006; Jiang, Chiu, et Hsu 2011). There is therefore a direct relationship between the capacity of TAP63 to regulate its target genes, and its capacity to be degraded (Ying et al. 2005).

In the male germline, studies in mice knockouts for *TP63* (-/-) reveal an increased number of gonocytes compared to wild type mice, and adult *TP63* (+/-) male mice also show more gonocytes with abnormal morphologies (Petre-Lazar et al. 2007). Interestingly, it is the γ isoform that seems most expressed in the quiescent period following the fetal mitotic phase of gonocytes. More recent work (Beyer et al. 2011) has identified an N-terminal *TATP63* isoform (*GTATP63*) with an extended 5' region which expresses itself specifically in the male germline. This extended region is encoded in one exon (U1) that is expressed from a third promoter located upstream of promoter P1 and P2. This isoform, called *GTATP63*, results from the integration of a retroviral LTR sequence upstream of the *TP63* locus in hominid. In a way similar to the genomic integrity control provided by the TA isoform, cisplatin treatment of cultured cells leads to increased apoptosis when *GTATP63* is expressed. This places GTAp63 as both the male equivalent of TAp63's oocyte protection role, and also as a possible testicular tumour suppressor. It is however not clear

whether the activation of GTAp63 relies on the same model as the activation of TAp63 in oocyte.

VI. Pathological involvement of p63:

VI.1. p63 associated human syndromes

Just as the homozygous knockouts of *TP63* in mice lead to severe, mainly ectodermal, phenotypes, heterozygous mutations in the *TP63* gene have been associated with six different syndromes in humans that mainly present ectodermal defects in a general sense. These syndromes combine defects falling into one or several of the following categories: ectodermal dysplasia, orofacial clefting and limb malformation (**Figure 8**). Ectodermal dysplasia consists in the absence or malformation of ectoderm derived structures such as skin, hair, teeth and sweat glands, Ectrodactyly is a malformation of the hands or feet linked to the absence of one or several digits, and orofacial clefting is the incomplete closure of facial structures during development.

- EEC: The main syndrome associated with *TP63* mutations which combine all three types of developmental defects. **Ectrodactyly**, **Ectodermal dysplasia** and **Cleft lip/palate**
- AEC: **Ankyloblepharon** is characterised by the adhesions between upper and lower eye-lids, **Ectodermal defects** and **cleft lip/palate syndrome**. It is also referred to as Hay-Wells syndrome.
- RHS: **Rapp Hodgkin syndrome** encompasses ectodermal dysplasia and **cleft lip/palate syndrome**. It is therefore quite close to the AEC.
- LMS: **Limb mammary syndrom** is mainly represented by **Ectrodactyly** and defects in the development of the mammary glands and nipples. The ectodermal defects can be present but are generally mild.
- ADULT: **Acro-dermato Ungual-lacrymal-tooth syndrome**. ADULT defects are mainly Ectrodactyly, Ectodermal dysplasia
- SHFM4: **Non syndromic Hand Foot Malformations** are mainly associated with Ectrodactyly

Mutations specific to each of these syndromes almost always fall within p63 functional domains (van Bokhoven et McKeon 2002). EEC mutations typically fall within the DNA binding domain whereas AEC mutations are usually located in the SAM domain. This suggests that symptoms observed in EEC are provoked by defects in p63's ability to

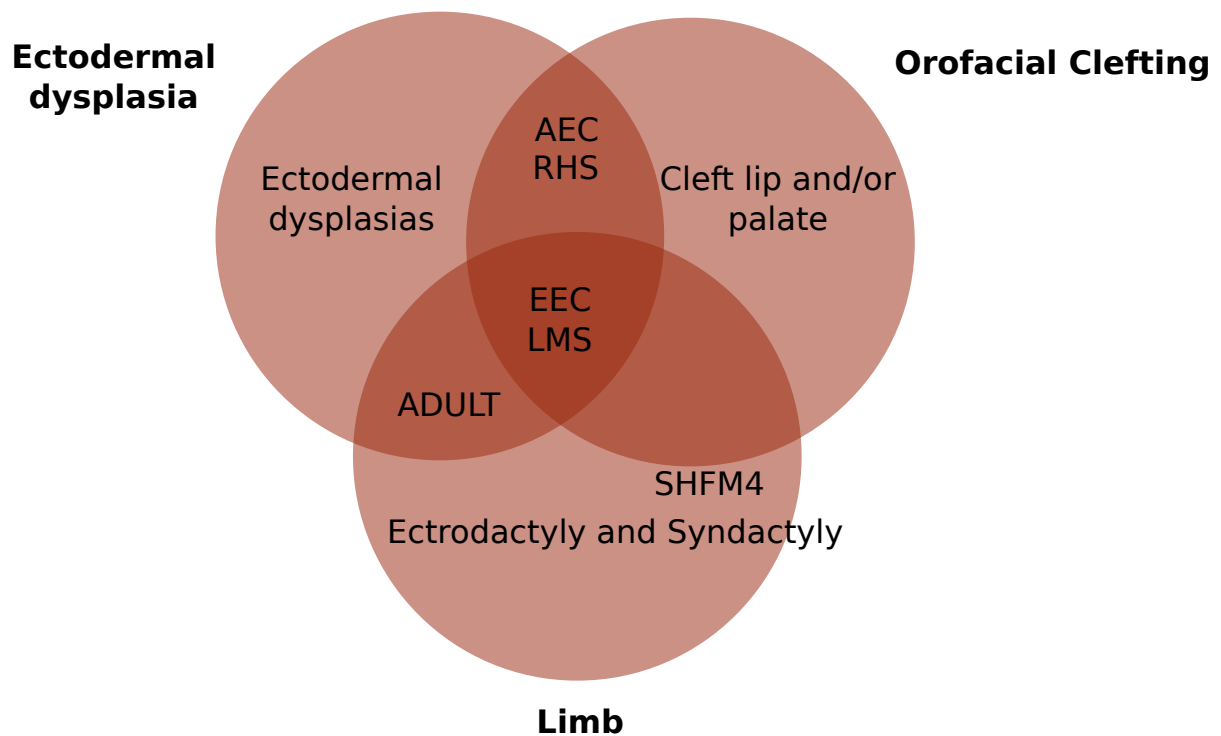


Figure 8. TP63 mutations and overlapping human syndromes.

The association of orofacial clefting with Ectodermal Dysplasia (ED) and limb malformations are the three hallmarks of *TP63* dependent developmental defects that are differently combined in different *TP63* dependent syndromes.. Ectrodactyly, Ectodermal Dysplasia, and Cleft lip/palate syndrome (EEC), Limb Mammary Syndrome (LMS), Ankyloblepharon-Ectodermal defects-Cleft lip/palate (AEC), Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT), Rapp-Hodgkin Syndrome (RHS) and non-syndromic Split Hand/Foot Malformation (SHFM4). (adapted from Rinne et al. 2006)

bind to its target sequences, whereas those observed in AEC must be the result of defects in interaction between p63 and its partners. Mutations in LMS have been found to affect the TID, which could indicate that poor p63 regulation is involved. However, a regulatory effect of the TID on Δ NP63 isoforms has not been proven as it has been for the TAP63 isoforms (Gebel et al. 2020). As ectodermal p63 functions are dependant on Δ N isoforms, the effect of these mutations could therefore be linked to defects in the recruitment of p63 partners known to interact with the TID such as HDACs (Ramsey et al. 2011). SHFM mutations are mostly found within the DBD, but also within the TID. Altogether, it is more than likely that there is overlap in target genes affected in each of these conditions, with specific differences likely stemming from modified interactions between p63 and its partners.

VI.2. Cancer

Since p63 is related to p53, the most infamous tumour suppressor, there has been a vast amount of work performed in the last twenty years to characterise its participation in cancer. Unlike *TP53*, *TP63* is very rarely mutated in cancers. However, based on the TCGA pan cancer atlas, *TP63* amplification is the most common alteration and happens in 1/3 of lung squamous carcinoma cases, 1/5 of oesophageal adenocarcinomas and 1/6 of cervical squamous carcinomas, head and neck squamous cell carcinomas or ovarian serous cystadenocarcinomas (**Figure 9**).

VI.2.1. p63 as a biomarker

Many studies have been performed to assess p63 expression in a wide variety of cancers and normal tissues. Often these studies focus on one particular type of cancer, with one particular antibody, therefore overseeing any specific isoform involvement. Recently, novel standardised work has been done in order to have a clear picture of p63 expression across all cancers (Steurer et al. 2021). Unsurprisingly, p63 expression is almost exclusively found in tumours derived from normal p63-expressing tissues. These are, for instance, squamous cell carcinomas of all locations, thymic tumours, urothelial carcinomas and basal cell carcinoma. p63 positive cells have however also been occasionally found in tumours deriving from p63 negative tissues of origin, such as gastric cancers, pancreatic cancers, and endometrial and ovarian cancers. In none of these cases was p63 expression associated with a worse outcome. p63 loss in cancers which typically

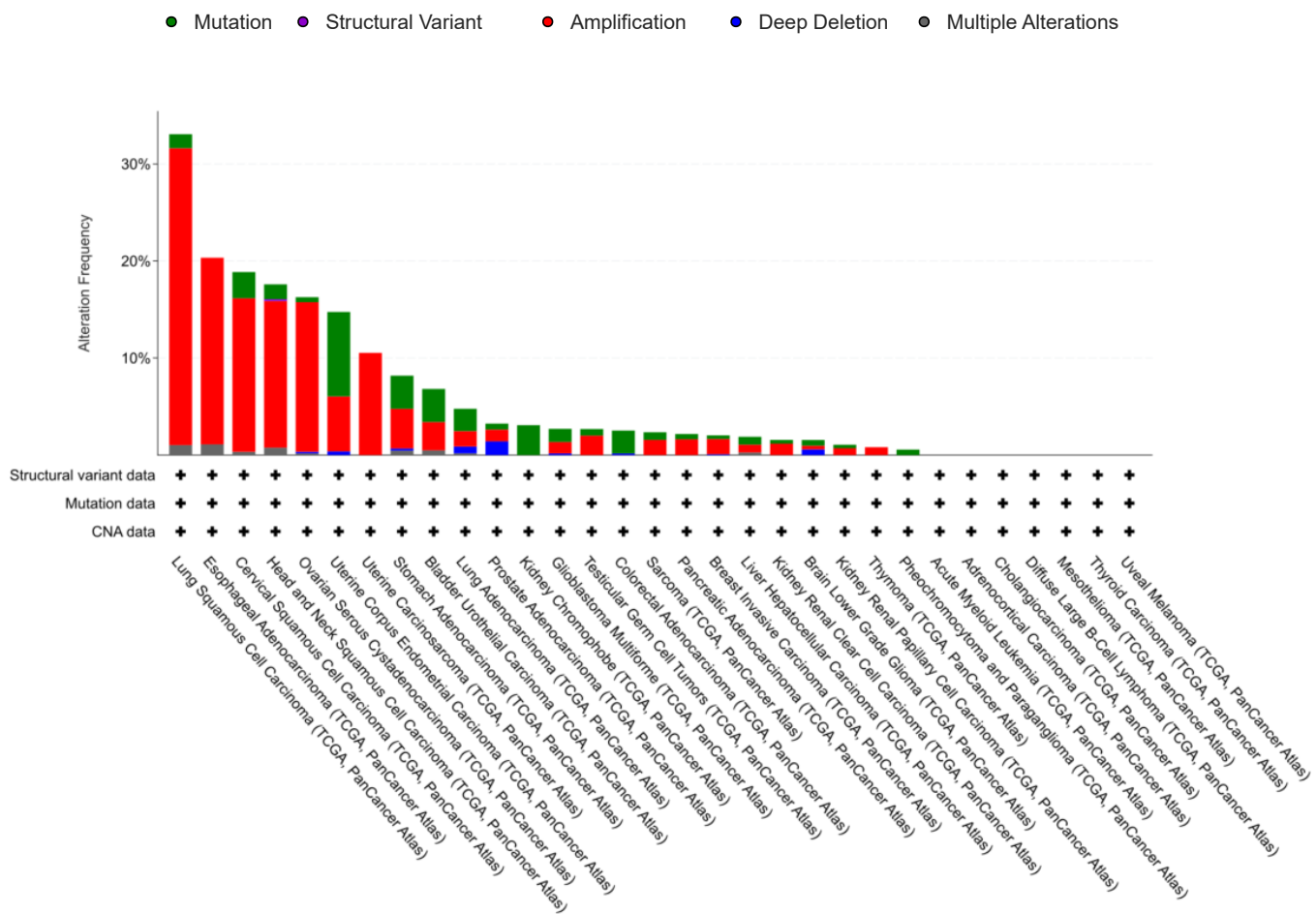


Figure. 9 : Alterations of the TP63 gene in Pan Cancer data (<https://www.cbioportal.org/>)
 Distribution of amplifications, mutations, structural variants and deletions in different cancer types.

express it, on the contrary, is frequently associated with poor outcome. This has been described in bladder cancer (X. Lin et al. 2014), oesophageal and laryngeal squamous cell carcinomas (Y. Takahashi et al. 2006; Borba et al. 2010), Head and neck squamous cell carcinoma (Lakshmanachetty et al. 2019a), and could also be the case in breast cancer (Xiaojuan Wang et al. 2002). Besides the predictive potential of its loss, p63 is also very useful in differentiating different types of cancer within a tissue, or distinguishing metastatic from local tumour cells. In lungs, for instance, p63 is useful for distinguishing adenocarcinomas (p63⁻) from squamous cell carcinomas (Bishop et al. 2012a).

VI.2.2. Tumour suppressor and oncogenic roles of p63

Despite being mainly expressed in cancer cells corresponding to normal p63 expressing cells, there is still a basis for evaluating how p63 can actively contribute to the disease. Indeed, if *TP63* is rarely mutated in cancers, *TP63* overexpression and/or amplification has been described in squamous cell carcinomas of various origins (Sniezek et al. 2004; Massion et al. 2003; DeYoung et al. 2006; Hibi et al. 2000; Tanière et al. 2001; Yamaguchi et al. 2000). In spite of its overexpression, loss of *TP63* is often associated with disease progression and poor outcome (Barbieri et al. 2006; Tucci et al. 2012; Lakshmanachetty et al. 2019a). Overall, investigated p63 functions in cancer can be broken down into three broad categories: metastasis and the Epithelial Mesenchymal transition (EMT), apoptosis, and proliferation.

- Metastasis and the EMT

EMT is a differentiation process promoting cell motility and invasive properties. These properties are the consequences of specific gene expression program that rely both on transcriptional and post-transcriptional regulators. EMT will be introduced in more in detail in the next chapter.

In Hs578T breast cancer cells which lack *TP63* expression, forced expression of $\Delta NTP63\alpha$ leads to the inhibition of cellular outgrowth in a matrigel matrix. A similar decrease in invasiveness was observed when $\Delta NTP63\alpha$ was expressed in lung cancer cell lines H1299 (nscl carcinoma) and A549 (adenocarcinoma). In this particular study (Miranti 2009), no effect was noted on EMT markers N-cadherin, E-cadherin and vimentin. Upon transfection with vectors encoding a mutated DBD version of *TP63*, these effects were not

found. The inhibition of invasiveness by Δ Np63 α is therefore mediated by its DNA binding capacity. Gene expression profiling identified subsets of genes whose expression is modified by expression of either wild type Δ Np63 α , Δ Np63 α -(C306R) with a mutated DBD or Δ Np63 α -(C526W) with a mutated SAM domain. Some genes affected by wild type Δ Np63 α expression overlapped with genes regulated by either one or the other of the mutants. There was no overlap between genes regulated by expression of Δ Np63 α -(C306R) or by expression of Δ Np63 α -(C526W). Δ Np63 α 's transcriptional impact can therefore be split three ways: genes that require the DBD and SAM domain to both be functional, genes that solely need the DBD, and genes that solely need the SAM domain. This last category is likely comprised of genes targeted by other transcription factors which have affinity for p63. This work also identified CD82, a known suppressor of metastasis (Miranti 2009), as a target of WT Δ Np63 α in Hs578T cells as well as in HaCaT, FaDu and MCF10a cells (Wu et al. 2014).

In bladder cancer Δ Np63 α transcriptionally promotes mir-205 which in turn inhibits pro-EMT transcription factors Zeb1 and Zeb2. Knockdown of Δ NP63 α in bladder carcinoma cell lines leads to increased mesenchymal markers. SNAI2 (Slug), another pro-EMT transcription factor, is positively controlled in the tested cells, with levels decreasing upon knockdown and increasing upon overexpression of Δ Np63 α (Tran et al. 2013c). In prostate cancer, loss of *TP63* is accompanied by a decrease in mir-205 levels and an increase in cell migration and metastasis (Tucci et al. 2012). The mir-205 is also a target of Δ NTP63 β in highly metastatic oral squamous cell carcinoma (OSCC) SQUU-B cells, giving Δ NTP63 β anti-EMT properties (Hashiguchi et al. 2018). Indeed, Δ NTP63 β expression shifts the phenotype of SQUU-B cells from spindle like to cobblestone-like, promoting their growth and decreasing their migratory capacity. Δ NTP63 β expression is also accompanied by an increase in cytokeratins CK5 and CK14 expression and a drop in N-cadherin and Vimentin expression, with no effect on E-cadherin. Vimentin expression is associated with loss of p63 at the tumour front in HNSCC, and is of poor prognosis for the patients (Goto et al. 2014). Conversely, In HS-2 cells, another HNSCC cell line, depletion of Δ NTP63 by siRNA leads to a change in cell shape from polygonal to spindle-like, a decrease in epithelial markers CK5 and CK14 and an increase of mesenchymal markers Vimentin and N-cadherin. These Δ NP63 cells concomitantly gain migratory abilities and decrease their proliferation (Goto et al. 2014). In transformed and non transformed keratinocytes, Δ NTP63 α specific knockdown leads to loss of epithelial markers and a gain of mesenchymal markers. This is echoed by the gain of migratory ability upon Δ NTP63 α

knockdown in HaCaT-RG, SCC-6 and HEK cells (Barbieri et al. 2006). In EP156T prostate cancer cells, clones which have undergone EMT lose *TP63* expression. Re-expressing *TP63* changes their spindle-like morphology to a near-cobblestone appearance and limits their ability to migrate, without completely changing the cells back to an epithelial phenotype. Conversely, depletion of *TP63* in epithelial EP156T cells does not provoke a loss of epithelial appearance (Olsen et al. 2013).

Mice with a conditional epithelial knockout of *TP63* (*Krt14* driven Cre, loxp sites around the DBD encoding region of *TP63*) show increased tumour burden following exposure to 4-Nitroquinoline-1-oxide (4-NQO), a carcinogenic compound used to reproduce the effect of tobacco use. Knockdown of *TP63* in FaDu and Cal27 cells increases their migration. Xenografts using *TP63*-knockdown cells promotes lymph node metastasis compared to control cells. No evidence of EMT was found in these *TP63*-knockdown FaDu and Cal27 cells. Instead, it is proposed that *TP63* loss leads to the activation of the MAPK and STAT3 signalling pathways. *TP63* could control MAPK activation by binding to the promoter of *DUSP6*, a negative regulator of MAPK signalling. Treatment with Trametinib, a potent inhibitor of MAP and ERK kinases, prevented *TP63* knockdown from enhancing FaDu and Cal27 migration, and also prevented lymph node metastasis following xenografts in nude mice (Lakshmanachetty et al. 2019a). Common mutations activating PI3K, Ras and Her2 pathways promote metastasis by inhibiting $\Delta Np63\alpha$ in HaCaT, FaDu and MCF10a cells. This is likely achieved through phosphorylation of Foxo3a, which dictates its cytosolic location. Nuclear Foxo3a is capable of binding to the *TP63* gene in its promoter region as shown by Chip experiments (Hu et al. 2017a).

In FaDu (OSCC) and MCF10A (Breast cancer) cells, which naturally express p63, shRNA mediated knockdown of all *TP63* isoforms leads to decreased levels of MKP3 and expression of Erk1/2 targets MMP1 and MMP9. This is accompanied by a gain in cell invasion and migration, with a change in cellular morphology of MCF10a cells from cobblestone to spindle-like. In breast cancer cell lines which lack endogenous *TP63* expression (MDA-MB-231), transfection with $\Delta NTP63\alpha$ expression vectors leads to an increase in MKP3 expression. Expression of $\Delta NTP63\alpha$ prevents nuclear accumulation of Erk1/2 thus decreasing the expression of its pro-invasion targets MMP1 and MMP9. Furthermore, p63 binding to a predicted site in MPK3's promoter has been shown by chip experiments. By reintroducing specific murine *TP63* isoforms in *TP63* depleted cells, $\Delta Np63\alpha$ has been identified as the only isoform capable of rescuing the migratory

phenotype. TAp63 isoforms have no effect, and $\Delta Np63\gamma$'s effect is marginal. In mice, injection into the tail vein of MCF10a-NeuT cells leads to more frequent lung metastases when they are depleted in *TP63* compared to control shRNA cells. Therefore, there seems to be solid proof defending a protective role of $\Delta Np63\alpha$ against metastasis (Bergholz et al. 2014). According to another study of MCF10a cells, knockdown of $\Delta NTP63\alpha$ and β leads to an EMT like phenotypic change, whereas depletion of all isoforms did not effect their epithelial phenotype. Knockdown of *TP63* isoforms decreased the cell's proliferation in both cases. Oppositely overexpression of $\Delta NTP63\gamma$ selectively induced EMT in the context of a depletion of all *TP63* isoforms. $\Delta Np63\gamma$ therefore seems to have a pro-EMT role, which is counterbalanced by the abundant presence of $\Delta Np63\alpha$ (Lindsay et al. 2011a). $\Delta Np63\gamma$ has also been reported to promote EMT and invasiveness in an HPV E6/E7-expressing human foreskin keratinocyte (HFK) cellular model (Srivastava et al. 2017; 2018a).

Altogether, most studies indicate that $\Delta Np63\alpha$ acts against cellular migration, either by inhibiting the EMT or by specifically modulating actors of cellular adhesion. In certain circumstances however, $\Delta Np63\alpha$ could also enhance metastasis. Indeed, when exposed to TGF- β , $\Delta Np63\alpha$ promotes metastatic dissemination by inhibiting miR-527 and miR665 (Rodriguez Calleja et al. 2016). This could have a big impact in the case of metastasis of $\Delta Np63\alpha$ expressing cancer cells to the bone niche which is naturally rich in TGF- β . $\Delta Np63\beta$ could have a similar role as $\Delta Np63\alpha$ in protecting cells from EMT. On the other hand, $\Delta Np63\gamma$ could have pro-EMT properties, but considering this effect has not always been observed (Bergholz et al. 2014), further work needs to be performed.

- Apoptosis

p63 has been described as both an activator and a repressor of apoptosis.

In human Hep3B cells (hepatocellular carcinoma), transfection of *TATP63 α* induces apoptosis. Gene expression analysis by microarray showed an induction of death receptor genes as well as genes involved in intrinsic apoptosis triggering. In Saos2 cells (osteosarcoma), *TATP63 α* expression also triggers cell cycle arrest and apoptosis by activation of p21 and transcriptional activation of pro-apoptotic genes. *TATP63 α* sensitizes the cells to CD95, TNF, TRAIL, and mitochondrial induced apoptosis, and thus sensitizes cells to chemotherapeutic agents (Gressner et al. 2005).

Uveal melanoma cell lines have low *TP63* expression and low *PERP* expression. In particular, OCM-1 cells, which do not express P63 following loss of chromosome 3q, enter

apoptosis accompanied by *TP53* and *PERP* upregulation after *TP63* transfection. (Awais et al. 2016). It has been previously described that *PERP* is a transcriptional target of p63, and that p63 could cooperate with p53 and p73 in its activation following DNA damage (Flores et al. 2002). p63 interacts with HDAC1 and HDAC2 in SCC cells. p63 can therefore bind to its target genes and recruit HDACs to remodel the chromatin in a transcription-permissive manner. One such target is *PUMA* (Ramsey et al. 2011). In zebrafish, $\Delta Np63\alpha$ is required for ER-stress induced apoptosis in the epidermis, via transcriptional activation of *PUMA* and subsequent caspase-3 activation, independently of p73 and p53. (Pyati et al. 2011). p63 α could therefore promote apoptosis in ways both with and without cooperation of p73 and p53.

In HaCaT and NHEK cells, *TP63* depletion decreases caspase-8 levels whereas overexpression of $\Delta NTP63\alpha$ in NHEK transcriptionally activates caspase-8. UV-irradiation concomitantly decreases p63 and caspase-8. Knockdown of *TP63* by shRNA increases caspase-8 activation, and overexpressing $\Delta NTP63\alpha$ inhibits caspase-8 activation following UV-exposure. Binding of p63 to the caspase-8 promoter region has been confirmed by CHIP. Furthermore, the apoptosis inhibitor FLIPr decreases following *TP63* depletion and FLIPs (also anti-apoptotic) and FLIPr increase when $\Delta NTP63\alpha$ is overexpressed, including after UV exposure. *TP63* null mice have lower caspase-8 levels but higher activated caspase levels and lower FLIP staining in K14 positive cells (Borrelli et al. 2009).

In bladder carcinoma 5637 cells, $\Delta NTP63\alpha$ knockdown by siRNA leads to an increase in apoptosis and sensitizes the cells to DNA damage induced apoptosis (Lee et al. 2006). In FaDu and HaCaT cells, chemotherapeutic agents decrease $\Delta NTP63\alpha$ transcription levels. Depleting $\Delta NTP63\alpha$ in these cells by RNAi leads to sensitisation to apoptosis whereas overexpression of $\Delta NTP63\alpha$ confers resistance to Dox and CDDP induced apoptosis (Xiaorong Li et al. 2012).

In HNSCC JHU-029 (WT p53) and JHU-011 (p53 truncated) cells *TP63* (mostly $\Delta NTP63\alpha$) is more expressed than in primary human keratinocytes. ShRNA against *TP63* induces apoptosis in both cell lines, which is rescued by concomitant expression of shRNA resistant $\Delta NTP63\alpha$. The p63 knockdown triggers PMAIP1 (NOXA) and BCC3 (PUMA) expression. Co-depletion of *TP73* abrogates the onset of *TP63*-knockdown-triggered apoptosis. The function of p53 has no incidence on apoptosis induction in these cells. p73 has been previously described as an activator of PUMA (Melino et al. 2004). Furthermore, endogenous $\Delta Np63\alpha$ and TAp63 β physically interact in JHU-029 cells as demonstrated by

co-immunoprecipitation experiments. Activation of BCC3's promoter by TAp63 β is inhibited by the presence of Δ Np63 α . The inhibition could either be through competition for binding to the promoter, sequestration by formation of p63/p73 heterotetramers, or both. Some HNSCC cells do not express Δ Np63 α while still expressing TAp63 β . Their survival is proposed to be linked to increased levels of Bcl-2, which has an anti-apoptotic function. In JHU-029 cells, overexpressing Bcl-2 prevents p63 depletion-mediated apoptosis (Rocco et al. 2006)

The exact effect of p63 on apoptosis seems to be somewhat cell type-specific. At least some of the variability of effects likely comes from the presence or absence of other p53 family members, as well as potential mutations in p53. Indeed, many apoptotic genes possess response elements which can be targeted by several members of the family, which creates the perfect opportunity for competition. Apoptotic pathways are also notoriously sensitive to the cellular micro-environment, which is often neglected in cellular studies.

- Proliferation, survival and senescence

In oesophageal squamous carcinoma cells, *TP63* knockdown leads to a drop in proliferation. In these cells, *TP63* levels correlate with Akt activation (Ye et al. 2014). In murine keratinocytes, the overexpression of Δ NTP63 α creates a c-Rel nuclear accumulation by its physical association to Δ Np63 α 's C-terminus. This protects the cells from growth arrest signals and promotes their proliferation in an NF- κ B dependant manner (King et al. 2008). In mice keratinocytes, Δ Np63 α is also said to repress p16^{ink4a} and p19^{arf}, counteracting cellular senescence (Ha et al. 2011). Conditional depletion of *TP63* in mice leads to a cellular senescence increase and a reduction of their lifespan (Keyes et al. 2005). In HNSCC cell lines, the TCF3 transcription factor targets the *TP63* gene, stimulating its expression. In turn, the increase in Δ Np63 promotes cellular proliferation. In oesophageal and lung squamous carcinoma patients, *TCF3* and *TP63* levels are correlated, and increased *TCF3* expression has a negative impact on patient survival (Xu et al. 2021). Overall, these different studies place p63 as a signalling node which promotes proliferation and represses senescence, two properties which contribute to cancerous growth.

In summary, there is little doubt that *TP63* is important. The many different functions of *TP63* can be assigned to 2 categories, control of the genome integrity, functions that are primarily taken care of by TAp63 isoforms and control of epithelial homeostasis and differentiation that are mainly the consequences of Δ Np63 function. Many articles refer to the importance of the different C-terminal isoform of *TP63* as important for the function of either the TA or the Δ N but so far no references have described mechanisms that could control the accumulation of any of these C-terminal isoform. In this context my work was mainly aimed at the identification of regulators of the expression of those isoforms.

Maturation of pre-mRNA and its actors

I. Maturation of pre-mRNA

Although similar on many levels, gene organisation and expression varies greatly within the three great orders of life. Bacteria and Archaea generally opt for a streamlined approach with most of their mRNA being collinear to their genome. This allows for a smaller genome size per overall number of transcripts, thus optimising the speed of both replication and transcription. Since they are devoid of a nucleus, there is no physical barrier for access to the ribosomes, and translation into proteins can therefore happen straight after RNA synthesis. In eukaryotes, genes are usually discontinuous, alternating between sequences destined to be included in the mature mRNA, respectively named exons and introns. This was first discovered following the observation of adenovirus DNA-RNA hybrids. Indeed, depending on the maturation of the RNA, different DNA loops were formed to allow for base pairing of the corresponding sequences (Chow et al. 1977). This groundbreaking discovery earned Phillip A. Sharp and Richard J. Roberts the Nobel prize in physiology and medicine in 1993.

The presence of a nucleus in eukaryotes means that translation can never happen immediately after transcription. This opens up an extra window during which the newly synthesised pre-mRNA can sustain a series of modifications to become a mature mRNA. First, the newly synthesised RNA receives a 5' cap of 7-methyl-guanosine. Once capping is complete, the cap-binding complex signals for the beginning of splicing, the process by which introns are removed and exons are joined together. An endonucleolytic cleavage defines the 3' end of the RNA molecule which subsequently receives a poly-adenosine tail. These steps happen in a co-transcriptional context (Bird et al. 2005; Rigo et al. 2005; de la Mata et Kornblihtt 2006; Fong et Zhou 2001; Furger et al. 2002; Kornblihtt 2006; Laurencikiene et al. 2006; Listerman, Sapra, et Neugebauer 2006; McCracken et al. 1997; Rigo et al. 2005), and many of the factors involved can interact with the RNA polymerase II, resulting in mutual stimulation and regulation. By interacting with the c-terminal domain (CTD) of the polymerase, splicing factors can promote transcription elongation (Fong et Zhou 2001). Likewise, interaction with the RNA-pol II optimises the efficiency of the 3' processing of the pre-mRNA (Rigo et al. 2005), and in turn the definition of the last exon with following cleavage and polyadenylation participates in transcription termination (Dye et Proudfoot 1999).

I.1. Correct recognition of exons and intron boundaries requires specific sequences in specific places

In order to correctly process pre-mRNA into mature mRNA, the cellular machinery must be capable of distinguishing intronic sequences from exonic sequences. At the most basic level, this is ensured by four elements that define the substrates of the biochemical splicing reaction. The first is the 5' splice site (5' SS), consisting of the following consensus sequence: AG/GURAGU, with the GU dinucleotide marking the 5' extremity of the intron. Next, the 3' splice site (3'SS) is characterised by YAG/xx, the AG dinucleotide marking the 3' end of the intron. The 3'SS is preceded by a 10-20 pyrimidine stretch called the "polypyrimidine tract". The final element is the branch point sequence, a degenerate heptamer -YNYURAY- which must contain an adenine, located 20 to 40 nucleotides upstream of the 3'SS. **(Figure. 10)**

I.2. The challenge of large introns

As mentioned, setting the boundaries between internal exons and introns requires paired 5' and 3' splice sites. With eukaryotic intron size sometimes reaching beyond 100 kilobasepairs (L. Fedorova A. Fedorov 2005), how does this machinery efficiently recognize and join two splice sites so far apart?

The first question that has been addressed, is whether the splicing machinery preferentially defines an exon, or an intron first? In vertebrates, exons are usually much smaller than introns, and it therefore seems likelier that splice site pairing happens through exons than through introns. This model of assembly is called "exon definition" (Berget 1995; Robberson, Cote, et Berget 1990) **(Figure 11)**. Sequences presented in **(Figure 10)** are sufficient in the case of internal exons, however the recognition of the first exon and the last exon require other elements. Indeed, the first exon is flanked by the 7-methyl guanosine cap on one side and a 5'SS on the other. The cap is recognised by the cap-binding complex (CBC) which in turns stimulates the formation of the ribonucleoprotein particles required for splicing and stabilizes them at the 5'SS (Lewis et al. 1996). The last exon is flanked by a 3'SS and a poly(A) site. The presence of the terminal exon's 3'SS has a positive effect on cleavage and polyadenylation thanks to interactions across the exon (Nesic et Maquat 1994).

In cases where introns are relatively short, it is thought that splice sites are paired through the intron, referred to as "intron definition". Ancient eukaryotes such as yeast or drosophila have a majority of short introns (Goguel et al. 1993, Talerico and Berget 1994)

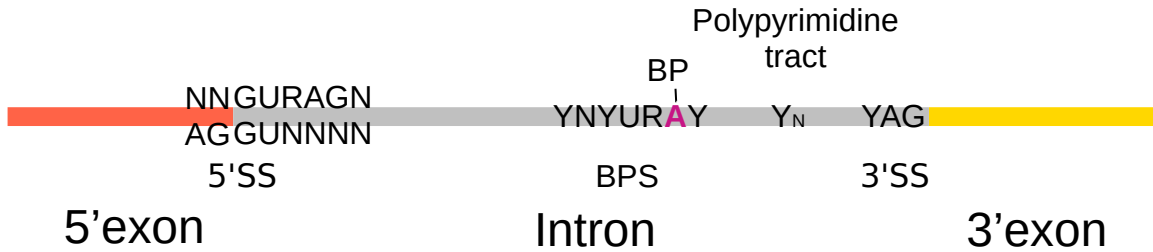


Fig.10. Schematic representation of splicing regulatory sequences within the Human pre-mRNA. Introns are minimally characterised by three short conserved sequences: a 5'splice site (5SS), a branchpoint sequence (BPS) containing the branch point (BP) and a 3'splice site (3'SS). A polypyrimidine tract is also present between the BPS and the 3'SS. The branchpoint adenosine is indicated in purple. (Adapted from Wilkinson et al. 2020)

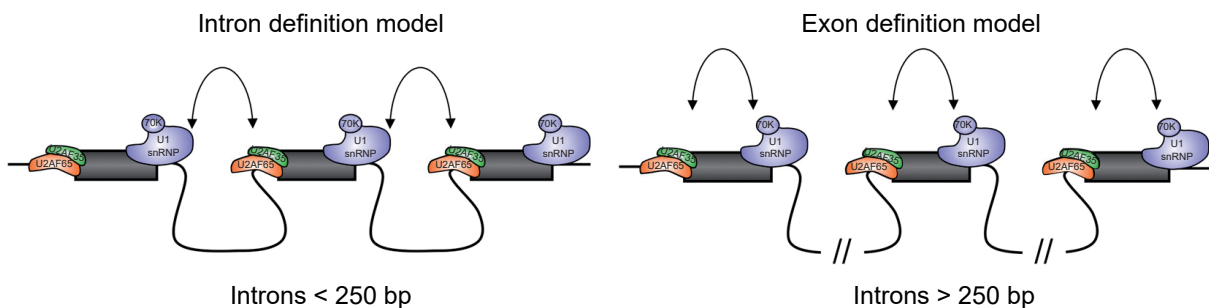


Fig.11. Exon and intron definition models.

The left panel depicts the intron definition model according to which pairing between the splice sites initially takes place across an intron when long exons are separated by introns shorter than 250bp.

The right panel depicts the exon definition model which happens with introns longer than 250bp, the splice sites initially communicate across the exons. (from De Conti, Baralle and Buratti. 2012)

and subsequently rely more on intron definition, whereas more recently evolved eukaryotes such as mammals have an abundance of long introns and thus heavily rely on exon definition. Both of these modes can however co-exist in any organism, and even within a single pre-mRNA. Regardless of intron size, initial exonic or intronic definition, the splicing reaction will always rely on the pairing of a 5'SS and a 3'SS through the processed intron. **(Figure 11)**

To deal with long introns, several processes have been described. Firstly, in *Drosophila*, a ratchet-like mechanism has been described whereby large introns are excised in a multi-step process. This recursive splicing process requires the presence of special ratcheting points (RPs) which act as both acceptors and donors. (Hatton, Subramaniam, et Lopez 1998; Burnette et al. 2005). In mammals, RPs are very rare, but the recursive mechanism could still be important while relying on different sequences, notably dispersed 3'SS and BPS dispersed throughout the intron (Hayashi et al. 2018; X.-O. Zhang et al. 2018). The second proposed mechanism for dealing with large introns is based on the presence of complementary sequences which favour the creation of stem-loop structures throughout the intron. In mammals, the frequency of such elements is much higher than in *Drosophila*, with most of the sequences being SINE or LINE elements. In primates, roughly 80% of the hairpin-forming structures were *Alu*-sequences (Shepard, McCreary, et Fedorov 2009). It is thus proposed that as retrotransposition contributed to expanding genomes, the repeated elements they introduced enabled a new and efficient means of regrouping 5' and 3' extremities of large introns, thus alleviating the selective pressure on RP sequences.

1.3. The spliceosome: where the magic happens

The splicing process requires the dynamic positioning and readjustment of 5 small nuclear RNAs (snRNAs) and roughly 100 proteins. These constitute the spliceosome. The five snRNAs are commonly named U1, U2, U4, U5 and U6. These interact with Sm, or L-Sm proteins to form the small ribonucleoprotein (snRNP) complexes of the same name (Will et Lührmann 2011). In some rare cases, the U1 and U2 snRNPs are substituted by snRNPs U11 and U12 resulting in a complex called the "minor" spliceosome, which mainly recognises non-canonical intronic AT/AC dinucleotides (Steitz et al. 2008). Here, I will focus only on the description of the major spliceosome. Far from being a static entity, the spliceosome goes through many different states as the pre-mRNA splicing reaction

progresses, through recruitment and release of partners and structural changes. These steps make up the splicing cycle as represented in **Figure 12**, which I will describe next.

Before any splicing reaction can occur, the different required sequences are brought together as the earliest form of the spliceosome: the E-complex. To form the E complex (E for early), the first step is the association of U1 snRNP with the 5'SS, which requires basepairing between the 5' end of U1snRNA and the exonic and intronic sequences of the 5' splice site. Besides binding of U1 snRNP to the 5' splice site, the E-complex also involves association of other proteins with the 3' splice site. Splicing Factor-1 (SF1) recognises the BPS, while U2AF subunits U2AF 65 and 35 recognize the polypyrimidine tract (Py-tract) and the 3'SS AG respectively. The assembly of these factors allows the successful bridging of the intron. The next event is the association of U2 snRNP with the branch point which is mediated through displacement of U2AF and SF1 generating the stable pre-spliceosome complex also referred to as the A complex. The U2snRNA-premRNA interaction requires basepairing between conserved nucleotides of U2snRNA and the branch point sequence leading to the formation of a helix in which the branch point adenosine bulges out. To create a complete spliceosome, the pre-assembled U4/U6-U5 tri-snRNP adds itself to the structure, and the pre-B complex is thus formed. The destabilisation of U1 basepairing to the 5'SS and subsequent basepairing of U6 to an intronic subset of 5'SS nucleotides allows the complex to rearrange itself, gaining splicing competence. This process requires RNA helicase activity, both to separate U1 from the pre-mRNA and to dissociate U4 from its base-paired partner U6. Prp28 insures the first step, eliminating U1 while Brr2 insures the second. The extensive basepairing between U6snRNA and U4snRNA is thus destabilised allowing the 3' end of U6snRNA to basepair with the 5' end of U2snRNA, still associated with the branch point. Concomitantly, U5snRNA basepairing with terminal nucleotides of the 5' exon is enhanced. The newly rearranged, catalytically active, spliceosome is now the B^{act} complex. ATP dependant changes in folding bring part of U2 closer to U6 and the bound pre-mRNA to form the catalytic site. The U1 and U4 snRNAs dissociate during the transition between the pre-B and the B^{act} complex. Thus, U1snRNA is involved in the early recognition of the 5'SS whereas U4snRNA is involved in the delivery of U6snRNP to the spliceosome and prevents premature activation of U6snRNA. Once everything is set for the branching reaction, the C complex is formed. There, the 5' exon is cleaved by a nucleophilic attack of the BP adenosine's free 2' hydroxyl group on the 5'SS guanosine's phosphate group, forming a looped structure known as the lariat (**Figure 12**). The first step of the splicing

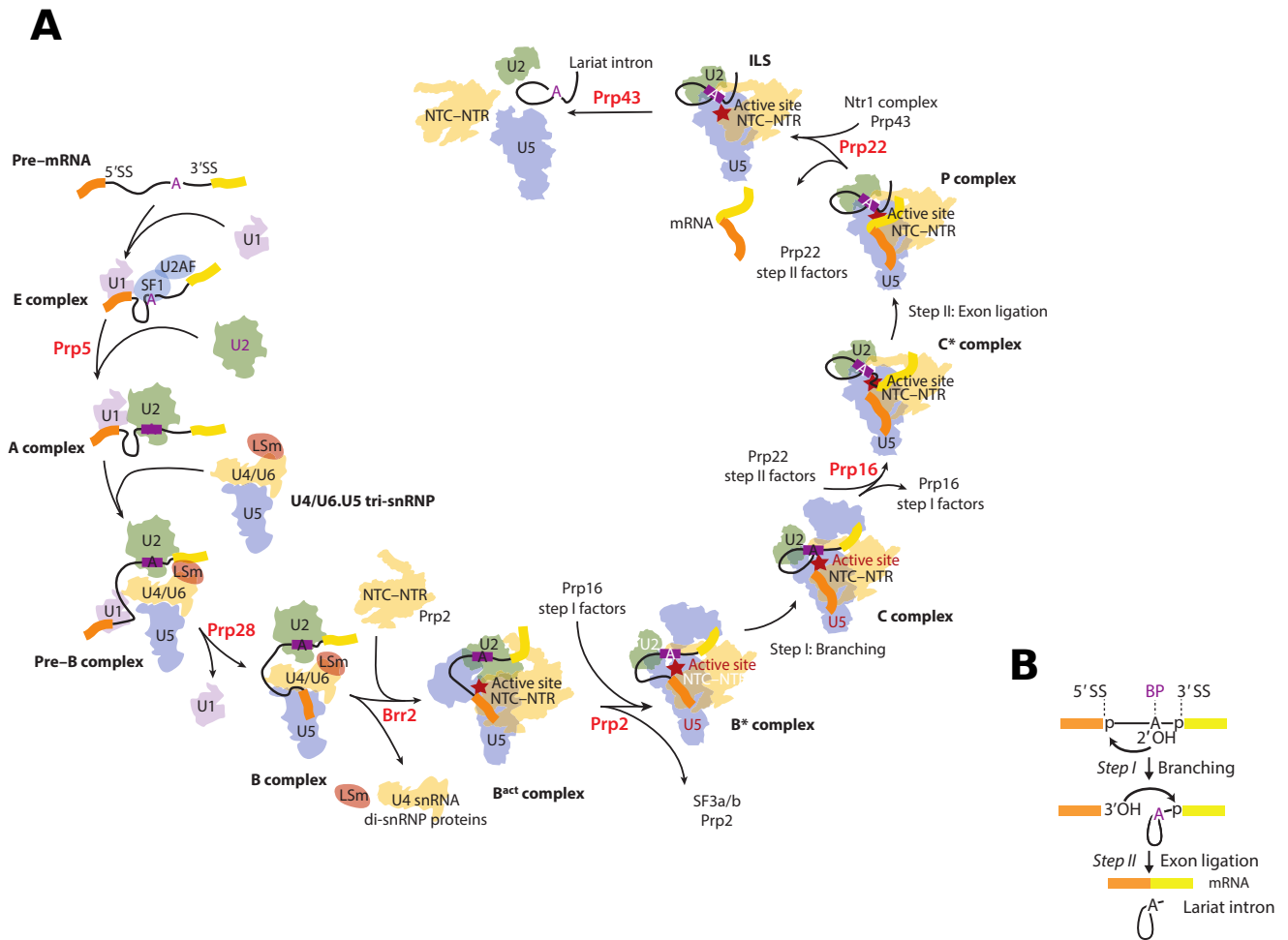


Fig.12 : The splicing cycle

A. The spliceosome is assembled in a highly ordered manner, activated to form the active site and remodeled extensively to perform the branching and exon ligation reactions, release the mRNA and disassemble. ATPases from the DEAD-box, DEAH-box and Ski-2 families depicted in red play crucial roles in this remodeling. **B.** Introns are removed following two trans-esterification reactions, branching and exon ligation, which are catalyzed at a single active site.

Abbreviations: BP branch point, ILS intron-lariat spliceosome, mRNA messenger RNA, NTC Prp-19 associated complex, NTR Prp-19 associated complex, snRNP small nucleolar ribonucleoprotein, SS splice site. (from Wilkinson et al. 2020)

reaction is then complete, with the spliceosome still attached to the 5' exon and the intermediate lariat. This intermediate complex is referred to as C*. Additional structural rearrangements are required to proceed to the second step. U5snRNA, already in interaction with the intronic part of the 5'SS, allows the second nucleophilic attack of the free hydroxyl of the 5'exon on the 3'SS guanosine's phosphate group, leading to the fusion of the two exons together (**Figure 12B**). This completes the formation of the P complex. Following the second step of the splicing, the snRNPs remain associated with the lariat whereas the exon-exon product is released. Finally the snRNPs are released from the lariat, which is debranched and degraded. With two exons now joined together, and a released lariat, the actors of the spliceosome may then be recycled for reuse in subsequent splicing reactions.

I.4. Cleavage and polyadenylation

With the 5' end of the pre-mRNA having received a 7-methyl guanosine cap, and the sequence contents of the future mRNAs established through splicing, the 3' end also needs to be taken care of. With the exception of cell-cycle regulated histone mRNAs, which adopt a 3' terminal stem loop structure after cleavage (Gallie, Lewis, et Marzluff 1996), the 3' extremity of mRNAs is matured through two sequential reactions: an endonucleotidic cleavage followed by the nuclear polyadenylation of the newly created 3' end. Cleavage and polyadenylation goes hand in hand with the definition of the terminal exon. Recruitment of the spliceosome and the cleavage and polyadenylation machinery into one macrocomplex allows for mutually beneficial interactions which optimise the efficiency of both reactions (Cooke et Alwine 2002).

Just like the splicing reaction, cleavage and polyadenylation requires well defined sequence elements. A cleavage site, generally in the form of a CA dinucleotide, is surrounded by two motifs: the polyadenylation signal (PAS), which is a hexanucleotide, usually AAUAAA, located 10 to 30 nucleotides upstream of the cleavage site; and a U or G/U-rich downstream sequence element (DSE) located roughly 30 nucleotides after the cleavage site. Supplementary regulation can be provided by a U-rich upstream sequence element (USE), located before the PAS, and a G-rich sequence following the DSE. (**see Figure 13**)

The sequences defining cleavage and polyadenylation sites are loosely defined. Because of the great length of eukaryotic genes, this means that besides the regular sets of PAS a pre-mRNA possesses, it usually also has a number of cryptic PAS. In case of

recognition, these would lead to premature cleavage and polyadenylation. To prevent this, an additional activity of U1 snRNP called telescripting is to bind to U-rich regions the RNA independently from the spliceosome, and acts as a protector against premature cleavage and polyadenylation (Berg et al. 2012; So et al. 2019).

The core machinery responsible for the cleavage and polyadenylation of pre-mRNA is exclusively constituted of proteins in mammals. Here, I will briefly describe the eight main actors presented in **Figure 14**.

- ◆ **Cleavage and Polyadenylation Specificity Factor (CPSF):** CPSF is constituted of five different subunits(CPSF-160, CPSF-73, CPSF-100, CPSF-30 and HFip1). The main subunit, CPSF-160, directly binds the **PAS**. CPSF-73 binds to the **C/A** cleavage site in a hexanucleotide dependant manner, and could be responsible for the endonucleotidic cleavage. CPSF-100 interacts with itself and with other proteins in the processing complex. CPSF-30 binds to the **U-rich** sequence and coordinates the assembly of other factors; its zinc finger motifs make it another possible candidate for the endonuclease activity. HFip1 interacts with both PAP and other CPSF subunits, it contacts the RNA on the U-rich upstream auxiliary sequence.
- ◆ **Cleavage stimulation factor (CstF):** CstF-64 binds to the RNA on the G/U rich DSE by its conserved RRM motif, placing it physically downstream of the CPSF complex. It also interacts with CstF-77 and symplekin via a hinge domain, and with CF-1A via its carboxy-terminal domain. CstF-77 likely acts as a dimer, and can interact with the carboxy-terminal domain of the RNA-polIII along with CstF-50.
- ◆ **Mammalian cleavage factor I (CF Im):** CF Im is a heterodimer made up of a small subunit of 25kDa, and a choice of three large subunits of either 59, 68 or 72 Kda. Together, they interact with the RNA in a sequence-dependant manner, aiding in the definition of the polyadenylation site.
- ◆ **Mammalian cleavage factor II (CF IIm):** CF IIm is also composed of two subunits. HPcf11 can bind both the RNA-pol II and the RNA and subsequently influences transcription termination and the release of the 3'end processing factors from the polIII HCip1 interacts with CF Im and CPSF.
- ◆ **Poly(A) polymerase (Pap1p/PAP):** Pap1p is a required enzyme for cleavage and polyadenylation. The length of the poly(A) tail is dictated by the nature of its

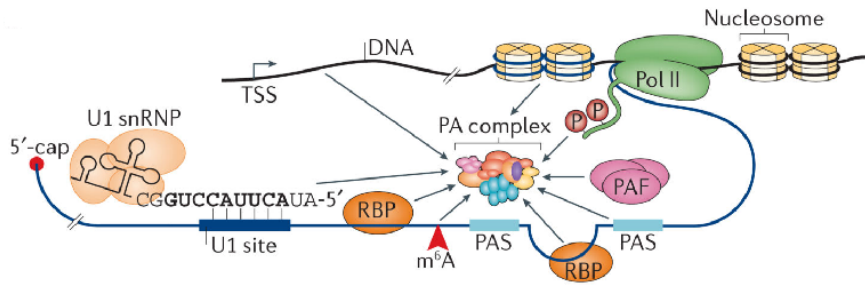


Fig.13 : multiple factors affect definition of Cleavage and polyadenylation sites

Multiple cues are factored in during alternative polyadenylation site choice. The gene's promoter, recruitment of regulatory proteins to the polyadenylation complex, the RNA pol II and its interaction with the RNA pol II associated factor PAF, various RNA binding proteins bound to the nascent transcript, presence of N⁶-methyladenosine (m⁶A) and inhibition by U1 snRNP all influence the final outcome of alternative polyadenylation..

(from Tian and Manley. 2017)

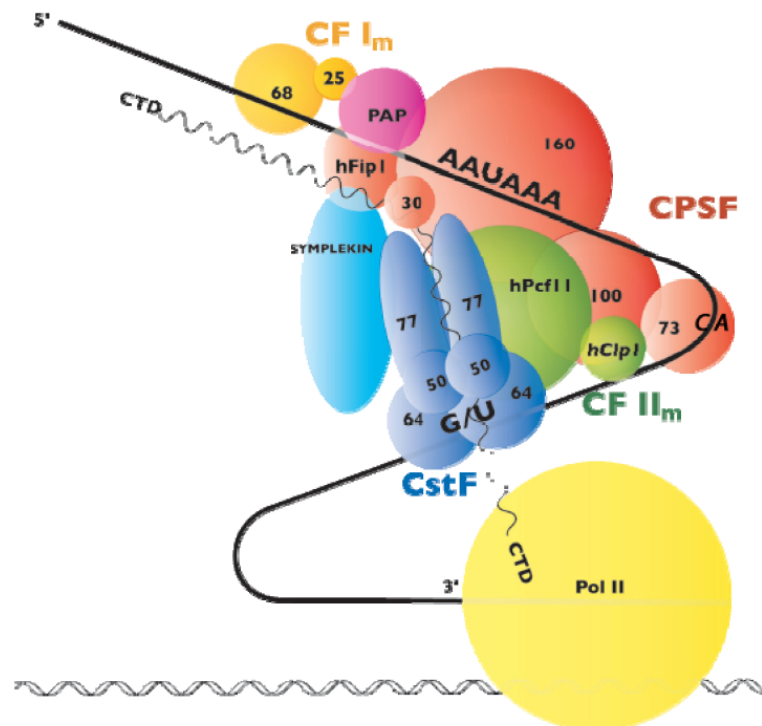


Fig.14. Mammal cleavage and polyadenylation complex.

The cis-regulatory elements, GU rich sequence (G/U), the cleavage site (CA) and the hexanucleotide (AAUAAA) are presented with the trans-acting factors of the cleavage and polyadenylation machinery. The protein composing the CPSF complex are shown in red, the Cstf complex in blue, CFIm and CF II_m in orange and green respectively.(from Mandel et al. 2008).

interactions with the other 3' end processing factors, with a notable interaction between its C-terminal domain and hFip1 and CPSF-160.

- ◆ **Poly(A) binding protein (PABP):** PABP are involved in the reaction while the addition of the poly(A) tail occurs and are essential for determining the length of the poly(A) tail. They also increase the efficiency of the polyadenylation reaction. PABP binds to adenine residues via strongly conserved residues in its two RRM domains.
- ◆ **Symplekin:** Symplekin likely acts as the main scaffold protein of the 3' end processing complex. It stably binds to both CPSF and CstF.

The precise order in which the described actors of cleavage and polyadenylation come together as a stable cooperative complex on the pre-mRNA is not fully elucidated. We do however have many clues to put the puzzle together. First of all, CF Im has a very high affinity for RNA and promotes the assembly of the complex (Rüegsegger, Blank, et Keller 1998). It is therefore thought that CF Im could be the first factor in place. Purification of splicing complexes led to the co-identification of both CF Im and CPSF. The two factors must therefore bind the RNA early on, which would make CPSF the second factor in place, bound to the hexanucleotide (Rappsilber et al. 2002). Then comes CstF, fixing itself to the DSE, and CF IIm joins in too (Neve et al. 2017). PAP recruitment is ensured by CPSF (Kaufmann et al. 2004). The whole complex stabilises itself, with extra help from symplekin.

Once the complex is in place, the catalysis of the endonucleotidic reaction can begin. This is likely performed by CPSF73 (Ryan, Calvo, et Manley 2004). The resulting 3'-OH of the upstream RNA can then be polyadenylated in a subsequent step ensured by CPSF, PAP and PABN1. Around 250 adenine residues are usually added and covered by Poly(A) binding protein nuclear-1 (PABPN1). When the poly(a) tail reaches its final length, the interaction between CPSF and the PAP ceases, marking the end of the reaction. On the other side of the cleavage, the downstream RNA fragment has a free 5' phosphate extremity and therefore gets degraded by Xrn2 (S. West, Gromak, et Proudfoot 2004). This degradation participates in the recycling of the RNA pol II.

II. Alternative splicing, alternative cleavage and polyadenylation, and their regulation

II.1. Alternative splicing

Since the generalisation of high throughput RNA sequencing, it has been estimated that more than 95% of Human genes are expressed as two or more mRNA isoforms (Ben-Dov et al. 2008). Alternative promoters, 5' and 3' splice sites and alternative cleavage and polyadenylation sites can be used in combination. The majority of described isoforms stem from cassette exon skipping, 3' or 5' alternative splice site selection, mutually exclusive exons or retained introns. **(Figure 15)**. As mentioned previously, the likelihood for an exon to be included in the mature mRNA is firstly defined by the strength of its splice sites. An exon with weak 5' and 3' splice sites followed by an exon with stronger splice sites is likely to be skipped. In a similar way, two 5' or two 3' splice sites of differing strengths may be close together, generating alternative donor or acceptor sites. At the most basic level, alternative splicing is therefore a stochastic process. The functions of alternative mRNA isoforms frequently diverge, and evolution selected strategies to control splicing in a direction that is favourable to the cell and ultimately to the organism. The regulation of these splicing events can be responsive to outside stimuli, such as signalling molecules or biophysical and biochemical changes, they can also be tissue-specific or change throughout the development and differentiation of a given tissue. Alternative splicing patterns are therefore adaptive to physiological conditions, allowing an organism to respond to changes in the environment by determining how its genome is expressed. Of course, there are many more alternative splicing events than there are different splicing regulators. Most splicing regulators are also not strongly tissue-specific. This means that for the most part, there is no singular responsibility between one regulator and one alternative splicing event. Rather, the outcome of alternative splicing is the net result of all the different activators and repressors bound to the pre-mRNA, but also other contextual elements such as changes in the RNA structure, chemical modifications or mutations in its sequence. On top of this, since 80% of pre-mRNA splicing happens co-transcriptionally (Girard et al. 2012), the speed of transcription can have a direct impact on which splicing sites get used. Faster transcription can favour the use of stronger splice sites, whereas a slower transcription rate gives a chance to weaker splice sites to be used. Epigenetic modifications can also influence the outcome of splicing both directly through chromatin-adaptor complexes, or indirectly by changing the local nucleosome distribution. Indeed, an

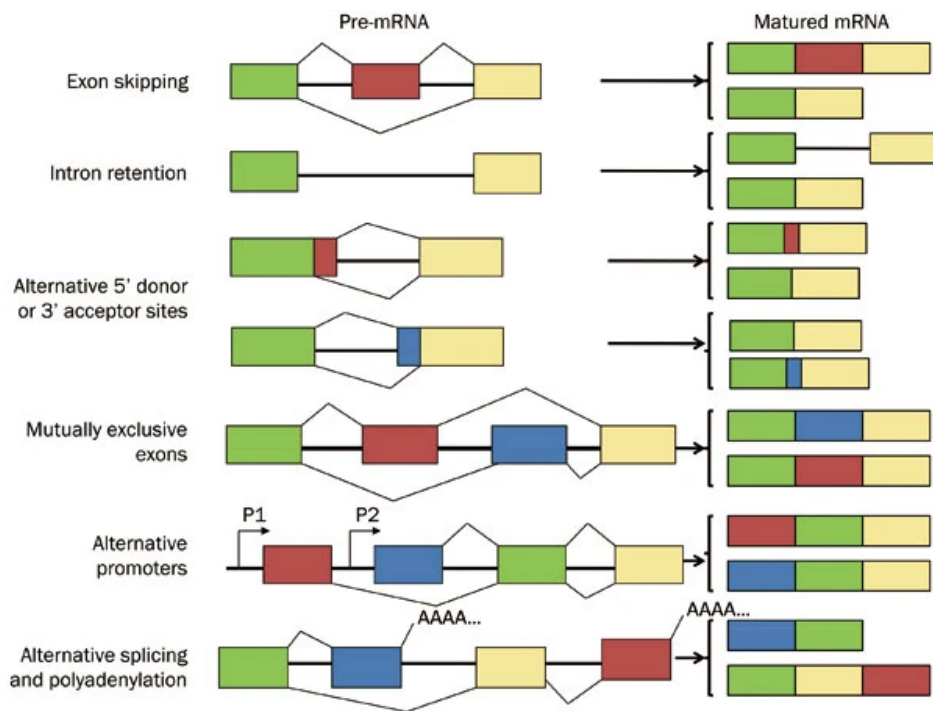


Fig.15. Schematic depiction of the most common alternative splicing patterns.

P1,P2 promoters, AATAA... cleavage and polyadenylation sites. pre-mRNA are shown on the left and maturated mRNA products on the right. Exons are represented as boxes and splicing events as broken lines joining two 5' and 3' splice sites (from Kai-qin Le et al. 2015).

increase in nucleosomes often leads to RNA polymerase II pauses, which create opportunities for splicing complexes to initiate (Luco et al. 2011).

The three previously mentioned splicing sequences, namely the 5'SS, BPS and the 3'SS, to which we could add the polypyrimidine tract and the AGEZ, are required for correct spliceosome assembly, but they are not sufficient to determine the outcome of alternative splicing. These sequences are degenerate, meaning that sequence variations create strong splice sites and weak splice sites depending on their relative affinity to the splicing machinery. The strength of the splice sites determines how likely an exon is to be included in the mature mRNA. Some exons are surrounded by splice sites so strong that they are constitutively included, while others are often skipped. The strength of the splice site, however, is not the be-all and end-all of exon use as there are additional sequences which recruit proteins capable of stimulating or inhibiting spliceosome assembly. These sequences are classed as intronic or exonic splicing silencers or enhancers, depending on their location and function (Figure 16).

Exonic splicing enhancers, or ESEs, typically stimulate the formation of the E complex by binding activator proteins. Intronic splicing enhancers work in much the same way, and tend to be localised close to the 5' and 3' splice sites where they help with the recruitment of splicing machinery. (S. Lin et Fu 2007). Negative splicing regulation is provided by exon and intron splicing silencers, usually by changes in conformation of the RNA or by steric hindrance that decrease interaction of activating factors with enhancer sequences. The proteins involved belong to the hnRNP family and SR proteins (Martinez-Contreras et al. 2007). These factors recognize specific intronic or exonic sequences which facilitate or inhibit, in a position-dependent manner, the recognition of neighbouring splice sites by the spliceosome machinery. Most proteins bind to single stranded RNA elements and changing local RNA structure can inhibit such binding. The availability of active splicing regulatory proteins in the cell can be influenced through modulation of expression levels, post-translational modifications or by control of their nucleo-cytoplasmic localisation.

The existence of splicing enhancers and silencers opens up a whole new possibility. Beyond dictating where exons and introns start and finish, they also make up a splicing code (Fu 2004; Baralle et Baralle 2018) by which pre-mRNA molecules can be matured into several different mRNAs by including or omitting different exons. This models of splice site selection places proteins as the main regulators. However, RNA itself could be

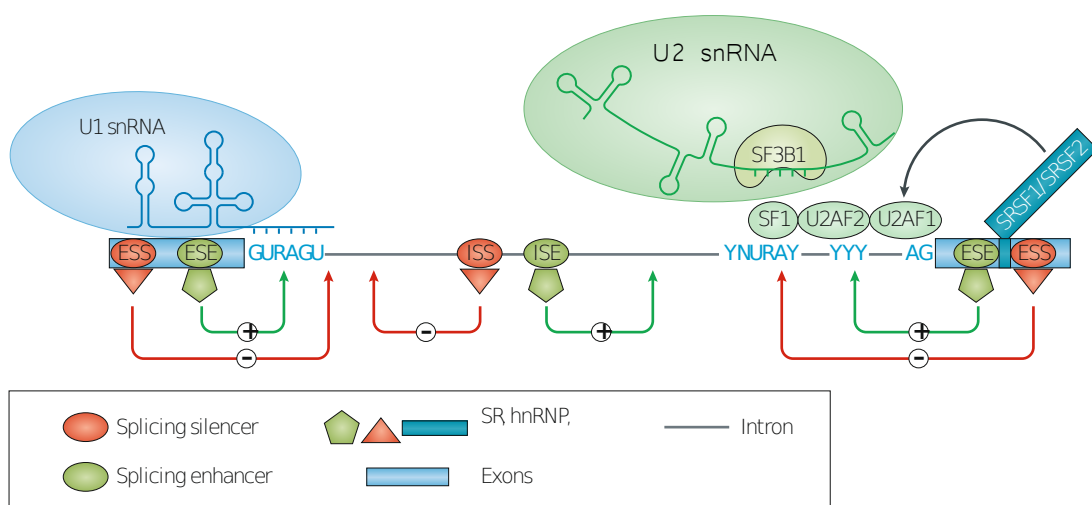


Fig.16. Cis-acting elements and trans-acting factors controlling alternative splicing.

RNA-binding motif (RBM) proteins, arginine-serine-rich (SR) proteins (including SRSF2) and heterogeneous nuclear ribonucleoproteins (hnRNPs) bound to exonic or intronic regulatory elements can promote or prevent the recognition of the 5' splice site by the U1 small nuclear ribonucleoprotein (snRNP) or of the 3' splice site by SF1, U2AF2, U2AF1, or U2 snRNP, thus affecting splice site choices and therefore alternative splicing decisions. (from Bonnal et al. 2020).

involved in this regulation and there is emerging evidence of a larger role of non-coding RNA in exon selection (Y. Yu et al. 2008; Khanna et Stamm 2010).

II.2. Alternative cleavage and polyadenylation

A large proportion of pre-mRNAs actually possess several sets of cleavage and polyadenylation sites, which can be alternatively used or skipped. When several polyadenylation sites are present in an RNA, the most distal sequence is usually the strongest (Moucadel et al. 2007). In the case where a proximal CPA site is used, the matured RNA will be devoid of all downstream exons or sequences following cleavage.

There are conceptually four different forms of alternative cleavage and polyadenylation (**Figure 17**):

- Tandem 3'UTR APA, when the APA sites are located within the same terminal exon, generating 3'UTRs of different lengths but sharing some identical sequences.
- Alternative terminal exon APA: in this case the different APA sites are located on different terminal exons. This influences both the length of the coding sequence and the nature of the 3'UTR of the transcript.
- Intronic APA: this mode relies on the recognition of cryptic ASA sites within introns, resulting in an extension of the otherwise non-terminal exon which precedes them, and a novel 3'UTR.
- Internal exon APA: an APA site is used within an internal exon, making it the terminal exon. A shorter transcript is thus created with a novel 3'UTR. If the exon is a coding exon, this may lead to non-stop decay (Vasudevan, Peltz, et Wilusz 2002).

The choice of polyadenylation site (PAS) during alternative polyadenylation can be influenced by various factors, including the gene promoter at the transcription start site (TSS); recruitment of factors that influence PAS choice; nucleosome density in the region around the PAS; RNA polymerase II (Pol II)-mediated transcription elongation by the Pol II-associated factor (PAF) complex; the function of various RNA-binding proteins (RBPs) associated with the nascent transcript; the presence of N^6 -methyladenosine (m^6A); and inhibition of polyadenylation by the splicing factor U1 small nuclear ribonucleoprotein (U1 snRNP). See the main text on cleavage and polyadenylation for more details.

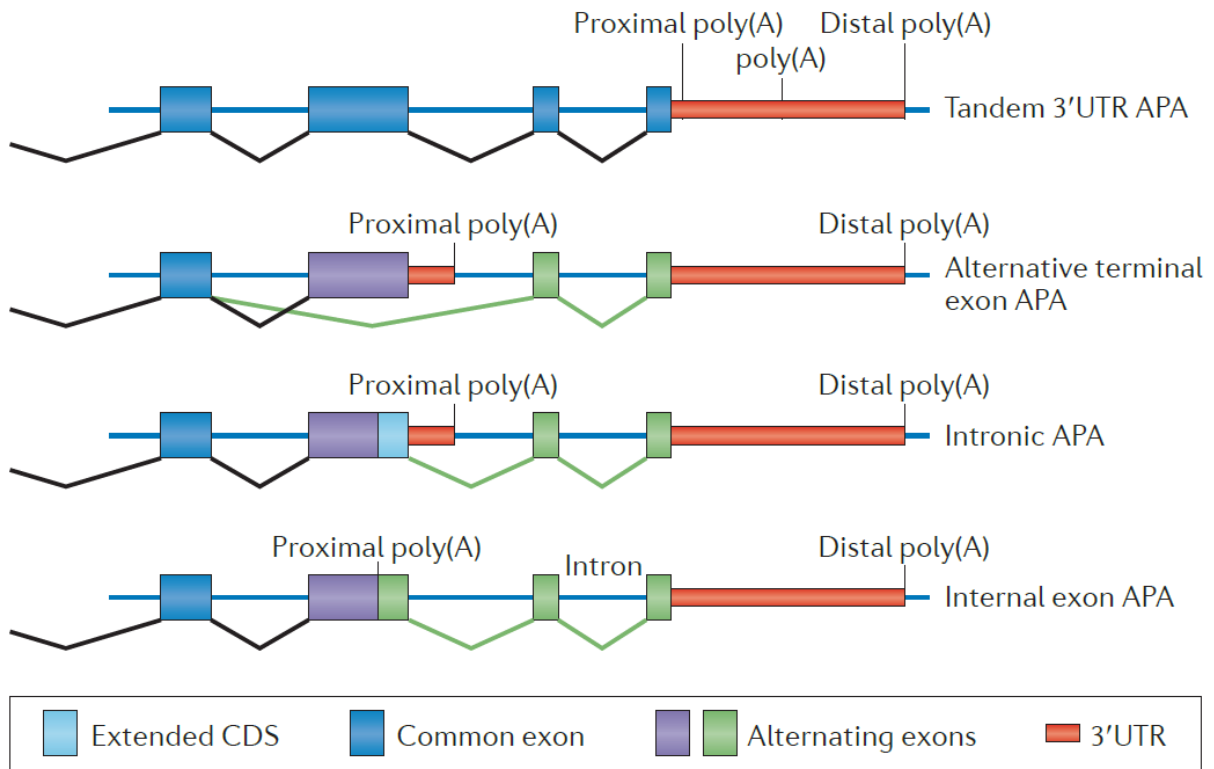


Fig.17. The four modes of alternative polyadenylation.

The combination of alternative splice sites and alternative cleavage and polyadenylation sites allows for multiple flavors of terminal exon definitions. (from Elkon et al. 2013).

II.3. Regulatory factors

The maturation of pre-mRNA by alternative splicing and alternative cleavage and polyadenylation offers the possibility for products from a single gene to have differing, context specific, functions or regulations. For this to work, however, the splicing machinery must receive specific instructions at the right place and time. In higher vertebrates, there are only a handful of tissue specific RNA binding proteins, and so it is proposed that the regulation is ensured by a combinatorial effect of varying levels of ubiquitous proteins. These signals are what constitute the cellular “splicing code”.

In a broad sense, any protein which possesses a domain capable of interacting with RNA, and therefore forming RNPs, can be classed as an RBP. The interaction with an RNA sequence or structure is generally provided by a globular protein domain of which there are three main kinds: the RRM, or RNA recognition motif, the KH or K homology domain, and the DEAD box helicase domains. Not all interactions between RNAs and RBPs are sequence or structure specific, a good example of these being the components of the exon junction complex. In this example, a complex of four subunits (eIF4A3, MAGOH, Y14 and BTZ) is recruited 20 to 24 nucleotides upstream of an exon-exon junction directly after splicing has occurred (Le Hir et al. 2000). This recruitment is performed by CWC22, an abundant constituent of the active spliceosome, by interaction with the eIF4A3 subunit (Steckelberg et al. 2012; Boehm et al. 2018).

The combination of RBPs bound to an RNA is modified as the pre-mRNA matures to an mRNA and is exported to the cytoplasm. Therefore, the RNP compositions evolve throughout the mRNA's life, and can therefore impact many different functions: capping, pre-mRNA maturation, nuclear export and mRNA localisation, protection or degradation, and even modulating RNA function through covering of regulatory portions, be they in UTRs or in coding sequences, and aiding in the apposition of epitranscriptomic marks. We may also consider the functional impact of the interaction between an RNA and a protein in the opposite way: some classes of RNA, such as lncRNAs, could just as well be regulating as being regulated by their cognate binding proteins.

In the context of this introduction, I shall focus on the two main families of RNA binding proteins involved in pre-mRNA splicing regulation : SR and hnRNP proteins.

II.3.1. The SR protein family

SR proteins are usually activators of splicing, with a particular affinity for ESEs. The members of the family are related by the presence of one or two amino-terminal RRM domains, and a carboxy-terminal RS domain. By phosphorylation of the Serine residues of the RS domain, SR proteins can be moved from storage in nuclear speckles to active transcription sites. Most of these factors are ubiquitously expressed but their relative abundance or activity may differ in different tissues and can be modulated by induction of specific cellular signalling pathways

SR proteins are mainly located in the nucleus but some (SRSF1, 3 and 7) are also exported to the cytoplasm where they aid with mRNA export. This process couples the nuclear export of an mRNA with its proper splicing and 3' end processing (Müller-McNicoll et al. 2016), as well as with mRNA decay and translation (Sun et al. 2010; Sanford et al. 2004).

There are three proposed modes through which SR proteins could positively influence splicing. The first mode consists in stimulating the recruitment of U1 or U2AF when bound to an ESE. This could provide an extra positive signal for otherwise weak 5' and 3' splice sites. The second mode relies on the competition between the effects of SR and hnRNP proteins: by binding to the ESEs, SR proteins could inhibit the binding of hnRNPs to them, therefore promoting exon usage. The third and final mode by which SR proteins promote splicing is by creating a web of protein interactions which bridges the intron and contributes to the physical grouping of 5' and 3' splice sites.

II.3.2. The hnRNP protein family

Members of the hnRNP family (heterogeneous nuclear RiboNucleoProtein) owe their RNA binding capabilities to three sorts of domains: an RRM domain, in most cases, and sometimes either K Homology domains (KH) or Arginine-Glycine-Glycine domains (RGG). HnRNPs are present throughout the nucleus, ready to bind to nascent transcripts. They are capable of binding in a non-specific manner to RNA, but in the case of splicing regulation, they usually bind with high affinity to splicing silencer sequences.

II.4. Functional and pathological results of aberrant splicing

There are several ways in which mutations can be pathological by modifying splicing. (**Figure 18**). First of all, mutations can happen within a cis acting region. This can effect either the core splicing sequences or the regulatory elements. Mutations in cis

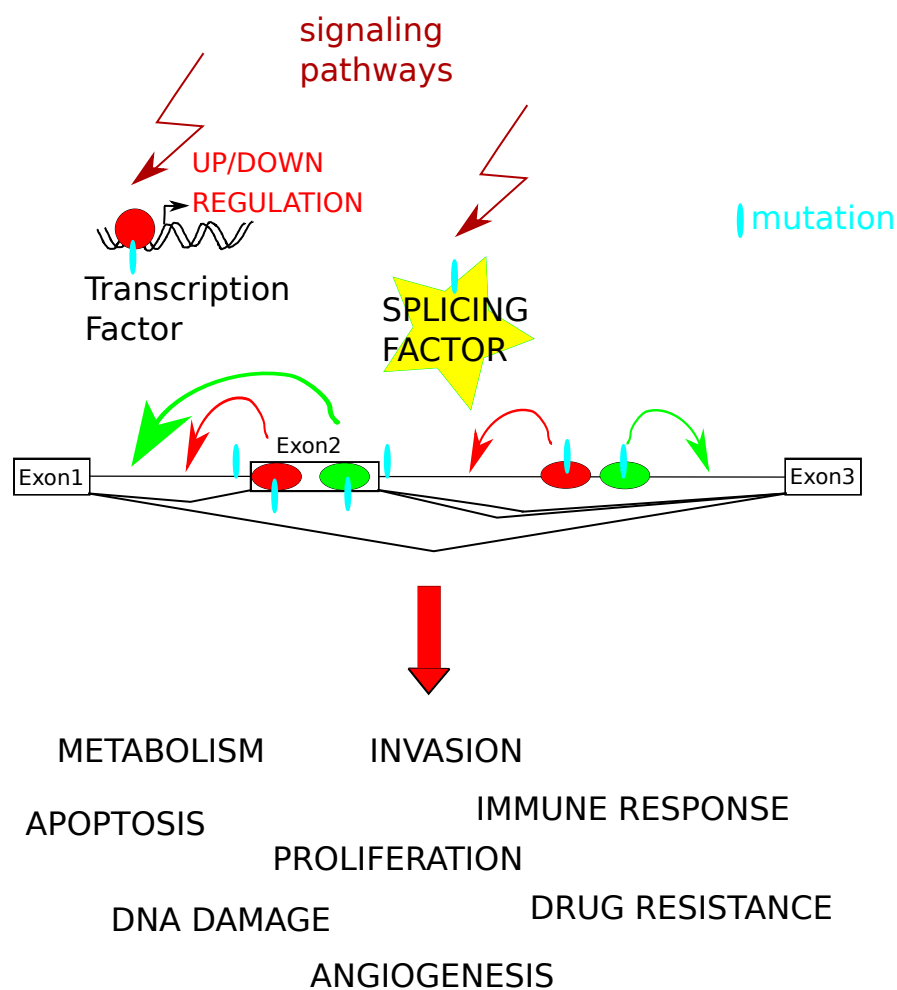


FIG. 18: Different entry points and outcomes of altered splicing decisions.

Alternative RNA splicing enables the production of distinct RNA isoforms from a single gene, providing transcriptional plasticity by controlling which RNA isoforms are expressed at a given time point in a given cell type. Defects in alternative splicing are frequently found in human tumours and result either from mutations in splicing-regulatory elements of specific cancer genes or from changes in the regulatory splicing machinery. Alterations of the splicing regulation may benefit cell proliferation or migration, or unable escape from cell death. Green: ESE or ISE, red: ESS or ISS

regions can also lead to splice site creation or disruption, either modifying exon boundaries or generating intron retention. These events very often introduce premature stop codons, leading to the degradation of the mature mRNA by nonsense mediated decay (NMD). These mutations therefore result in a loss of function of the mutated allele. There can also be trans effects of a mutation if it directly disrupts a splicing actor, either by direct mutation of the gene encoding the factor or by mutation affecting its expression level. In this case, the resulting disease can be due to the accumulation of many aberrant splicing events. In both trans and cis cases, the mutations can lead to the expression of either dysfunctional proteins, or functional proteins at abnormal levels. In a more indirect manner, mutations can happen in non coding genomic regions, resulting in either cis effects by alteration of the regulatory role of 5' and 3' UTRs or by altering the function in trans of lncRNAs and other non coding RNAs, which could involve mechanisms such as sponging of splicing factors or scaffolding roles during the establishment of splicing complexes.

II.5. Therapeutic strategies to modulate pathological splicing

Of the known genetic causes of disease, 15% are attributed to pre-mRNA splicing defects (Krawczak, Reiss, et Cooper 1992). In some cases of deleterious splicing, therapeutic action is possible. Antisense oligonucleotides targetting regulatory regions, or directed against aberrant splice sites, can lead to either the skipping of a particular splicing event or the degradation of a pathological mRNA. Therapeutic splicing modulation in pathological cells can be achieved by either fine-tuning the activity of splicing regulators or by precisely targeting a single spliced isoform. A good example of a splicing modulator is Spliceostatin A (Corrionero, Miñana, et Valcárcel 2011). This drug inhibits pre-mRNA splicing *in vitro* and *in vivo* by binding to SF3b, a protein component of U2 small nuclear ribonucleoprotein (snRNP), which is essential for recognition of the pre-mRNA branch point (**Figure 12A**). However, only a fraction of splicing events are affected. Differences in base-pairing strength with U2 snRNA in this region lead to different sensitivities of 3' splice sites to the drug. Through splicing modulation, Spliceostatin A impacts the expression of key regulators of cell division, including cyclin A2 and Aurora A kinase, which can contribute to the anti-proliferative activity of the drug.

Considering the importance of phosphorylations in activating and repressing proteins involved in splicing regulations, several kinase-targetting drugs have been developed (Ohe et Hagiwara 2015). NB-506, for example, inhibits the phosphorylation of SF2/ASF by topoisomerase I, preventing the splicing of pre-mRNAs containing SF2/ASF

target sequences (Pilch et al. 2001). Another example, TG003, inhibits the kinase activity of Clk kinases, potent regulators of the SR proteins (Muraki et al. 2004). This drug has shown therapeutic potential in the treatment of cystic fibrosis by limiting the inclusion of a pseudo-exon (Shibata, Ajiro, et Hagiwara 2020), in Duchenne muscular dystrophy to promote the skipping of a mutated exon (Nishida et al. 2011) and in anhidrotic ectodermal dysplasia with immunodeficiency, also by preventing the inclusion of a deleterious pseudo-exon (Boisson et al. 2019).

These small molecules often lack specificity, and their exact mechanisms of action are not always well understood. RNA-based therapeutics offer the potential to target any molecule (Kole, Krainer, et Altman 2012; Havens et Hastings 2016). Short antisense oligonucleotides targetting splicing are referred to as splice switching antisense oligonucleotides (SSOs). Depending on the chosen target location, they can either inhibit RNA:protein or RNA:RNA interactions. Thanks to their high specificity, SSOs can be used to negatively impact the inclusion of a sequence by binding locally to a splice site or a enhancer/silencer. This is useful to prevent the use of cryptic splice sites, for suppressing or introducing frameshifts and premature stop codons, or to direct splicing towards beneficial rather than detrimental isoforms. SSOs can also be directed against silencer sequences, which promotes the use of specific exons. This can be used as a compensatory mechanism in the case of mutations that weaken important splice sites (Havens et Hastings 2016). Beyond splicing, antisense oligonucleotides are also potent in targetting pathological cleavage and polyadenylation events (Vorlová et al. 2011).

The most successful example of the power of sequence targetted therapy is in the treatment of spinal muscular atrophy (SMA). SMA is an autosomal-recessive monogenic disease caused by mutations or deletions in the SMN1 gene (Lefebvre et al. 1995). Patients present with motor neuron degeneration that leads to progressive muscle weakness and, in severe cases, respiratory failure and death. Disease severity is inversely correlated to the abundance of functional SMN (Mailman et al. 2002; Wirth et al. 2006), a ubiquitously expressed protein involved in pre-mRNA splicing, snRNP biogenesis, transcription, stress response, apoptosis and axonal transport. In theory, the lack of SMN1 gene expression can be compensated by expression of its paralogous gene SMN2. SMN2's coding sequence diverges from SMN1 by a single translationally silent substitution in exon 7 (c.840C>T). Unfortunately, this substitution is sufficient to provoke skipping of exon 7 in most SMN2 transcripts (**Figure 19**) and leads to the production of a truncated mRNA, SMN2 Δ 7. SMN Δ 7 codes for an SMN protein isoform that is unstable and does not

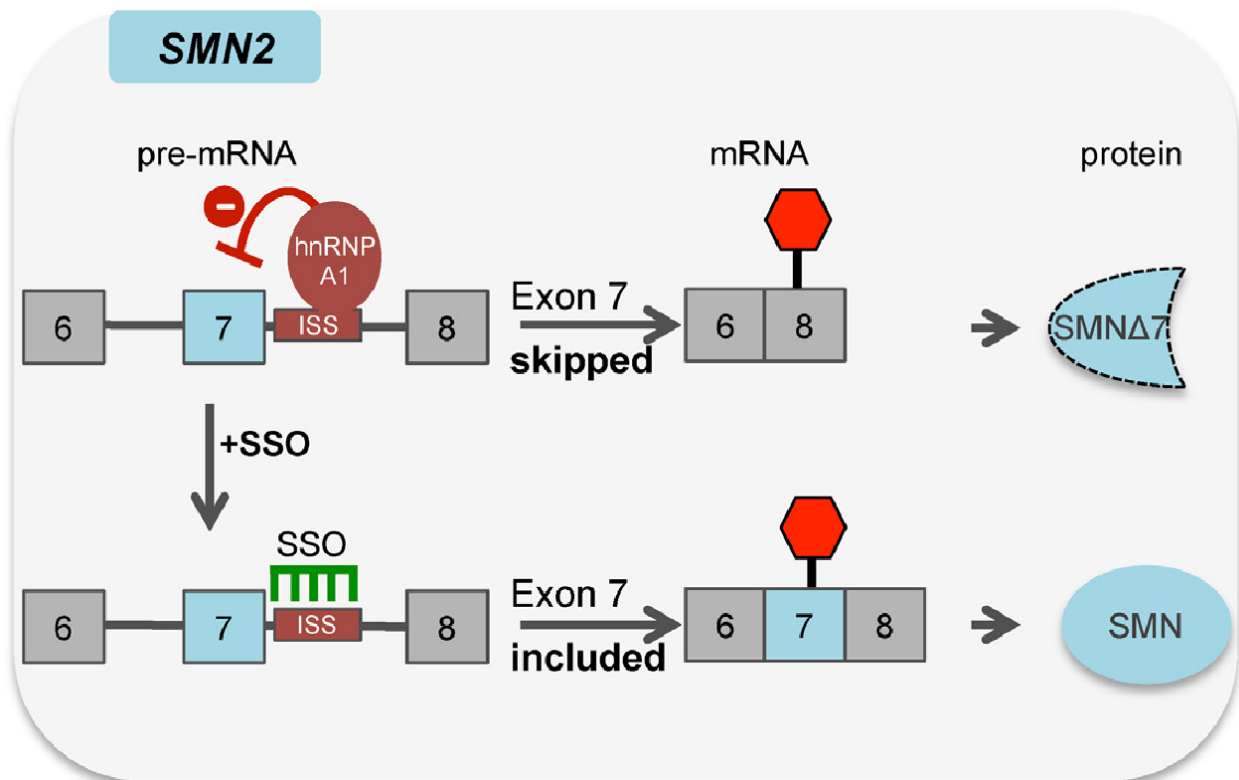


Fig.19. Modulation of splicing by SSOs in spinal muscular atrophy.

Schematic representation of disease associated splicing in Spinal Muscular Atrophy (SMA) within the SMN2 pre-mRNA, and the antisense oligonucleotide targeting strategy used to therapeutically influence splicing. (from Havens and Hastings. 2016).

function in the same manner as the full-length SMN isoform. Therapeutic efforts have therefore focused on correcting this splicing event. An antisense oligonucleotide derivative, SPINRAZA (nusinersen), has been approved for use on pre-symptomatic newborns and young children. Its binding to SMN2 pre-mRNA removes hnRNPA1 from the cis-RNA silencer flanking the 5'SS of SMN2's exon 7. The exon 7 can therefore be included, leading to the production of functional SNM protein (Hua et al. 2007; Beusch et al., s. d.; Haché et al. 2016; Foust et al. 2010; Finkel et al. 2017; Mercuri et al. 2018). Though nusinersen provides an appreciable therapeutic gain, it requires frequent injections, and merely delays the onset of the disease. Other therapies are therefore being developed. RISDIPLAM (Evrysdi), a small molecule targetting SMN2 splicing has now also been authorised for clinical use. RISDIPLAM binds to the 5'SS of SNM2's exon 7 and promotes its inclusion. Studies performed on SNM-C5, a small molecule similar to RISDIPLAM, suggest that the stimulation of exon 7's inclusion is based on the stabilisation of a free adenine at its 5'SS, increasing the strength of the splice site by promoting binding to the U1 snRNP(Campagne et al. 2019).

III. Focus on the polypyrimidine tract binding protein (PTBP1) hnRNP

During my thesis work, one particular hnRNP has waltzed into the limelight: PTBP1. The PTB family actually has three members sharing strong sequence conservation: the almost ubiquitous PTBP1, PTBP2, expressed primarily in neuronal tissue, and PTBP3, expressed in some immune cells. Binding of PTB proteins to RNA is made possible by the presence of four RRM domains, two of which are capable of interacting with each other, creating a loop like structure. As their name suggests, they share the capacity to bind to pyrimidine-rich sequences, with a proposed consensus of repeated UCUU or CUCUCU sequences(Oberstrass et al. 2005; Upadhyay et Mackereth 2020). Of course, the simplicity and *in vivo* abundance of these consensus sequences make it very difficult to accurately predict where PTB proteins will actually bind.

The abundance of PTBP1 in cells is determined through an elegant NMD-dependant feedback loop. By exclusion of exon 11, the PTBP1 RNA suffers a frame shift which subsequently leads to its degradation by NMD. In *Xenopus laevis*, exon 11 inclusion is stimulated by ESRP1(Méreau et al. 2015). When PTBP1 levels reach a certain threshold, the PTBP1 protein binds to PTBP1 pre-mRNA and represses the exon 11, leading to a decrease in its own expression(Wollerton et al. 2004). In a similar manner,

PTBP1 negatively controls PTBP2 expression by promoting the skipping of a cassette exon, leading to a frameshift and subsequent NMD (Boutz, Stoilov, et al. 2007; Makeyev et al. 2007; Rachel Spellman, Llorian, et Smith 2007).

PTBP1 levels are also subjected to regulation by miRNAs. During neuronal differentiation, mir-124 inhibits PTBP1 which allows PTBP2 expression (Makeyev et al. 2007). During myoblast differentiation, muscle-specific microRNA miR-133 expression is associated with the reduced level of PTBP1 (Boutz, Chawla, et al. 2007). In human pancreatic islets responding to the presence of high levels of glucose, mir-133a is induced leading to lower PTBP1 levels and lower insulin biosynthesis rates by the effect of mRNA destabilization (C. Wang et al. 2008).

PTBP1 is involved in many steps during the life of its mRNA targets with described roles in their translation, splicing, stability and localisation (Sawicka et al. 2008). These different functions are possible since PTB proteins possess both a nuclear localisation signal (NLS) and a nuclear export signal (NES) and can therefore shuttle between the nucleus and the cytoplasm (Kamath, Leary, et Huang 2001).

III.1. Role in translation

PTBP1 is capable of nucleo-cytoplasmic shuttling, and its function in internal ribosomal entry sites (IRES) mediated translation initiation of both cellular and viral mRNAs is extensively documented. Most translation initiation is mediated by the cap structure at the 5' end of the mRNA, which is recognized by the initiation factor complex eIF4F. The 40S ribosomal subunit interacts with eIF4F and scans along the RNA until it reaches the AUG start codon. In contrast, viral RNA from the picornoviridae family lack a cap structure and ribosomal recruitment occurs internally at highly structured elements known as IRES (Balvay et al. 2007). Interaction with the 40S ribosomal subunit is mediated by additional cellular factors, known as initiation of translation accessory factors (ITAFs) such as PTBP1. PTBP1 has been shown to be required in internal ribosome entry by a number of viral IRES (Martínez-Salas et al., s. d.). It has been proposed that PTBP1 may act as a chaperone to stabilize IRES structure to enable the recruitment of the ribosome and to position it at the start codon (Song et al. 2005). Outside of the viral context, PTBP1 also plays an ITAF role in the translation of several cellular factors. For example PTBP1 positively regulates the IRES activities of p53 isoforms, relocating them from the nucleus to the cytoplasm during stress conditions (Grover, Ray, et Das 2008).

PTBP1 protects transcripts from NMD degradation by displacing the RNA helicase UPF1 from its target sites. This inhibition is dependant on the enrichment of high affinity PTBP1 binding sites within a mRNA's 3'UTR, which enables protection even in the case of multiple UPF1 binding events (Fritz et al. 2020).

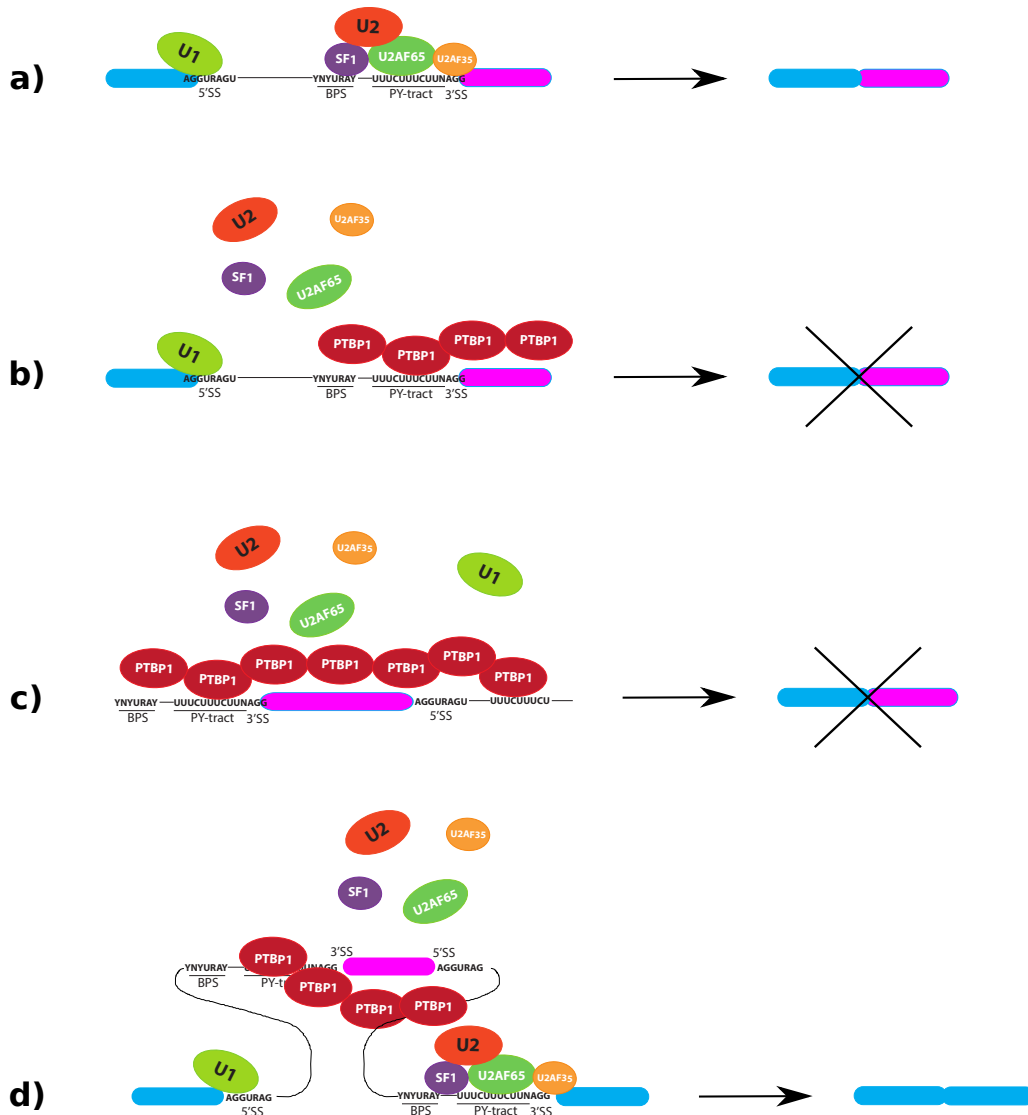
III.2. PTBP1's role in pre-mRNA splicing

During pre-mRNA splicing, PTBP1 often enters into competition with other regulatory proteins. In alpha-tropomyosin, PTBP1 competes with RBM4 for the binding to a CU-rich element. PTBP1 binding to this regulatory element inhibits the inclusion of muscle-specific exons in alpha-tropomyosin mRNA, whereas RBM4 stimulates them (J.-C. Lin et Tarn 2005, 4). PTBP1 competes with TIA to promote the skipping of exon 12 of Myosin Phosphatase Target Subunit 1 (MYPT1) by binding to a U-rich element downstream of the exon's 5'SS. Skipping exon 12 is one of the events necessary for smooth muscles to shift from a short-phasic to a fast-phasic contractile phenotype (SHUKLA et al. 2005). Competition between PTBP1 and TIA1 has also been described for the maturation of Fas (Apo-1/CD95) pre-mRNA. Fas can exist as a membrane bound receptor which triggers apoptosis upon binding of its ligand, or as a soluble protein which inhibits apoptosis by neutralizing Fas-ligand. The soluble form is produced following skipping of exon 6. PTBP1 inhibits the inclusion of this exon by binding to an exonic silencer and inhibiting the fixation of U2AF to the upstream polypyrimidine tract (Izquierdo et al. 2005). PTBP1 also represses the splicing of c-src's exon N1 onto exon 4 by preventing the binding of U2AF to the exon 4, which stalls the assembly of the pre-spliceosomal E complex (Sharma, Falick, et Black 2005).

So far, there have been three described modes by which PTBP1 can repress the inclusion of an exon:

- PTBP1 can bind its target pre-mRNA near the branch point, in the polypyrimidine tract, thus hindering its recognition by U2AF65 and the subsequent assembly of the splicing machinery. Good examples of this mode of action are the fibronectin EIIIB exon (Norton 1994) and exons 2 and 3 of the α -tropomyosin (Gooding et al. 2013; Mullen et al. 1991). Additional factors other than U2AF65 such as Celf1 could also antagonize the PTBP1 repressive interaction (R. Spellman et al. 2005). **(Figure 20. A-B)**

A.



B.



Fig.20. Mode of actions of PTBP1 on alternative splicing and cleavage and polyadenylation. A. Alternative splicing. In the absence of PTBP1, the two exons are spliced together (a). PTBP1 masking of a splice site (b) or covering the targeted exon (c) prevents its inclusion into the mature mRNA. PTBP1 multimerisation can exclude an exon from being spliced by creating a loop in the pre-mRNA (d) **B.** Cleavage and polyadenylation. (a) Normal assembly of the cleavage and polyadenylation factors onto the pre-mRNA. (b) PTBP1 masks the 3'SS and the CPA signals of a terminal exon, preventing its recognition by both splicing and CPA factors.

- When PTBP1 binding sites are dispersed around a regulated exon, PTBP1 proteins can act cooperatively to cover this exon, making it physically inaccessible to the splicing machinery. **(Figure 20. A-C)**
- If PTBP1 sites are present before and after a regulated exon, PTBP1 proteins can regroup and bind to each other, effectively creating a loop which excludes the exon from the remainder of the pre-mRNA and potentially bringing the surrounding 5' and 3' splice sites in a closer conformation. This mechanism was first described for the N1 exon of c-src. **(Figure 20. A-D)**

The action of PTBP1 in blocking exon and intron definition is a way to control many splicing fates. By binding to intronic or exonic elements, it can prevent the assembly of a productive spliceosome at the targeted exon, and can also prevent the exon definition by disrupting SR protein-dependent interactions across it.

As illustrated by the above examples, PTBP1 is one of the best described hnRNPs responsible for negative regulation of splicing by causing exon skipping. In some cases however, it can also act as an activator. In hnRNPA1 pre-mRNA, PTBP1 counteracts SRp30c's repressive function. SRp30c binds to a conserved intronic element (CE9) in hnRNPA1 pre-mRNA, resulting in the repression of its downstream 3'SS. PTBP1 inhibits this mechanism by fixing itself to a CU rich region two nucleotides upstream of SRp30c's binding site (Paradis et al. 2007).

III.3. *PTBP1's role in RNA polyadenylation*

During pre-mRNA maturation, besides its influence on splicing, PTBP1 is also capable of recognizing polyadenylation signals. Indeed, PTBP1 shares an affinity with CstF63 for polypyrimidine rich sequences. Several studies demonstrate that PTBP1 competes with CstF64 for binding to the pyrimidine-rich DSE. As a result of this competition, PTB inhibits mRNA 3' end cleavage, leading to a significant down-regulation of polyadenylated transcripts and a corresponding increase in unprocessed "read-through" transcripts (Castelo-Branco et al. 2004).

Sequences surrounding polyadenylation signals often include auxiliary cis-acting sequences, including U-rich upstream motifs and G-rich sequences (GRSs) positioned both upstream and downstream of the pA signal **(Figure 14)**. Both cis-elements have an enhancing function in 3' end formation through the binding of trans-acting proteins,

including PTBP1 (Danckwardt et al. 2007; Neve et al. 2017). PTBP1 can therefore affect the choice of polyadenylation sites leading to mRNAs with differing 3' ends, or proteins with different C-termini (**Figure 17**). Cis-acting elements promote the use of suboptimal polyadenylation signals and in the case of PTB, this regulation appears to be linked to its function as a splicing repressor. A well documented example of the involvement of PTBP1 in directing the choice of terminal exon is the calcitonin/calcitonin gene-related peptide (CT/CGRP) pre-mRNA. By binding to an intronic pseudoexon in CT/CGRP pre-mRNA, PTBP1 favours the inclusion of an alternative 3' terminal exon containing an alternative polyadenylation signal (**Figure 21**).

The calcitonin/calcitonin gene-related peptide (CT/CGRP) pre-mRNA presents two cell type-specific 3' terminal exons. Thyroid C cells use exon 4 as CT/CGRP's 3' terminal exon to produce the CT peptide. In neuronal cells, exon 4 is excluded, and inclusion of exons 3, 5 and 6 allows the production of a longer mRNA which codes for the CGRP peptide. The exon 4 is defined by a weak 3'SS and a weak polyadenylation signal. In order to be included, it therefore requires an intronic enhancer (ISE). This ISE is located downstream of intron 4 and contains a pseudo-5'SS, a pyrimidine rich sequence and U repeats (H. Lou et al. 1995). In vitro cleavage experiments have shown that the pseudo-5'SS and the pyrimidine rich sequence are both required for efficient cleavage and polyadenylation of exon 4. Moreover, the U rich sequence is said to contribute to exon 4's 3'SS recognition (H. Lou, Gagel, et Berget 1996; Zhu et al. 2003). Another regulatory sequence present within exon 4 enables the use of its 3'SS. These activator sequences are recognised by many factors including SR proteins (Hua Lou et al. 1998). In vitro, PTBP1 interacts both with the sequence located upstream of the polyadenylation signal and with the polypyrimidine rich sequence (**Figure 21.C**). In cells, overexpression of PTBP1 favours exon 4 usage by activating its cleavage step (H. Lou et Gagel 1999; H. Lou, Gagel, et Berget 1996).

PTBP1 is therefore a very potent regulator of alternative splicing and polyadenylation. Despite having been categorised for many years as ubiquitous, variation in PTBP1 levels are responsible for the regulation of many different types of post-transcriptional regulation and more specifically of alternative splicing and cleavage and polyadenylation events.

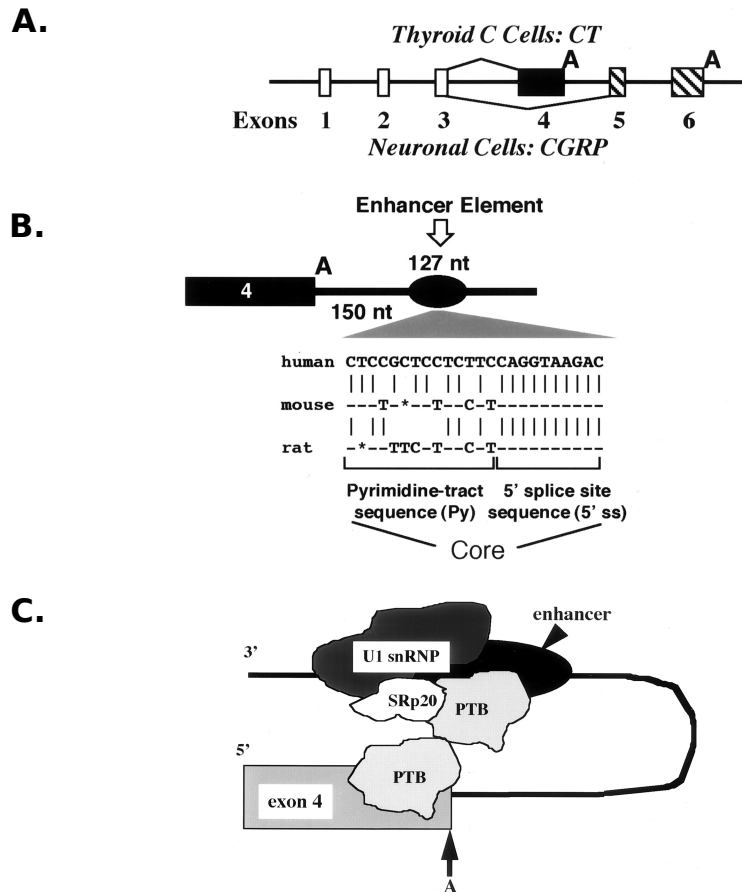


Figure 21 : PTBP1 controls the inclusion of a terminal exon in CT/CGRP RNA

A. CT/CGRP regulated internal terminal exon. Low quantities of PTBP1 in neuronal cells and higher levels in thyroid C cells. **B.** Polyadenylation enhancer element located downstream of the terminal exon. The location of the PTBP1 binding motif is shown. **C.** Model for PTBP1 mediated activation of CT/CGRP polyadenylation.

(from Lou et al. 1999)

III.4. PTBP1 and Cancer

PTBP1 expression may affect cancer initiation and progression by inducing aberrant splicing events in transcripts involved in many aspects of tumour progression. Cancer specific splicing events have been reported at the mRNA level in colon, bladder, and prostate cancer (Thorsen et al. 2008) Cancer specific alternative splicing events are also promising markers for cancer diagnosis (Venables 2006) as well as genetic diseases in a broader sense (Xiong et al. 2015).

For reasons still unknown, cancer cells usually shift their metabolism from oxidative phosphorylation to aerobic glycolysis, a process termed the Warburg effect. This shift is dependent on the splicing of two exons that allow the production of two isoforms of pyruvate kinase: PKM1 and PKM2. PKM2 is essential for glycolysis, as opposed to PKM1 which serves in oxidative phosphorylation. In cells, PKM1 and PKM2 are rate limiting enzymes and considered to be cancer hallmarks. Outside of its metabolic roles, it is suggested that PKM2 could promote cancer progression through its kinase activity, by activating the EMT program, proliferation, angiogenesis and drug resistance (Zahra et al. 2020). PTBP1 has been identified as a potent regulator of PKM2 (David et al. 2010).

Numerous reports highlight the involvement of PTBP1 in cancers of different origins and by modulating different cellular pathways. The following is a brief summary of some of their highlights.

PTBP1 is upregulated in breast cancer tumours and cell lines, and correlates with her2 expression, lymph node metastasis and disease stage. PTBP1 knockdown depletes the strong invasive capacity of MDA-MB-231 cells and in mice, tumours derived from PTBP1 depleted MDA-MB-231 show slower growth than control cells. Depletion of PTBP1 in patients presenting with Her-2 positive breast cancer therefore shows potential as a novel therapeutic approach (Xu Wang et al. 2018). In a similar way, PTBP1 is overexpressed in colorectal cancers, based on immunohistochemical staining of patient tumour samples. Again, PTBP1 depletion in a multitude of colorectal cancer cell lines increased their proliferation, but also inhibited their colony forming ability which translates to a limited capacity of anchorage-free growth. In mice, xenografts of HCT116 (colorectal carcinoma) cells have decreased tumorigenicity when depleted in PTBP1 (H. Takahashi et al. 2015).

In glioblastoma multiform tumours, PTBP1 could influence growth by promoting the production of the fibroblast growth factor receptor 1 (FGFR-1) with increased affinity for

FGF. In this model, PTBP1 binds to intronic splicing silencers upstream and downstream of the α exon, which then gets skipped, leading to the production of high-affinity FGFR-1. Furthermore, PTBP1 is dramatically increased during the malignant transformation of the glial cells (Jin et al. 2000). Analysis of migration rates of glioma cell lines U251 (glioblastoma) and LN229 (glioblastoma) cells has shown that knocking down both PTBP1 and PTBP2 impedes their motility (Cheung et al. 2009, 2).

In ovarian cancer, PTBP1, as well as SRp20, are overexpressed. They could promote exon skipping and partial intron retention in the ABC transporter “multidrug resistance protein 1” (MRP1) mRNA. Some of these splicing modifications in MRP1 promote resistance to doxorubicin, a drug frequently used in the treatment of recurrent or refractory ovarian cancer (Xiaolong He et al. 2004). During immortalisation of ovarian epithelial cell lines, PTBP1 levels increase. Its knockdown in A2780 cells impairs their proliferation, invasion and anchorage-independent growth, reminiscent of its effect on colorectal cancer cells (X. He et al. 2007).

Despite the convincing decrease in proliferation of many cell types, both normal and cancerous, PTBP1 overexpression has not been associated with a gain in proliferative or malignant abilities. Furthermore, in contrast with the previously cited examples, in PC-3M (prostate carcinoma) and T84 (colon adenocarcinoma) cells, depleting PTBP1 leads to a decrease in migratory abilities. (C. Wang et al. 2008). Considering the broad ranging targets of PTBP1, it is likely that its participation to cancer is strongly context and cell-type dependent. PTBP1's described involvement in different cancer types is summarised in **Table 2**.

Table 2. Roles of PTBP1 in various cancers (from Zhu et al. 2020)

Type of cancer	Mechanism	Function/effect	Reference
Melanoma	PTBP1/CD44v6	Migration of melanoma brain metastasis	Marzese et al., 2015
Hepatoma	miR137 and miR206/↓PTBP1/PKM1	Negative effects related to tissue characteristics and carcinogenesis	Taniguchi et al., 2018
Rhabdomyosarcoma	↓miR-133b/PAX3-FOXO1/PTBP1 and ↓miR-1 and ↓miR-133b/PTBP1	Positive regulation of cancer-specific energy metabolism	Sugito et al., 2017
Cervical cancer	HPV infection/PTBP1	Promotion of DNA replication and cancer cell proliferation	Xu et al., 2019
Acute myeloid leukemia with mutated nucleophosmin	PTBP1/PKM2/phosphorylation of Beclin-1	Autophagic activation and cancer cell survival	Wang et al., 2019
Anaplastic large cell lymphoma	PTBP1/PKM2/STAT3	Transcription of genes involved in cell survival and proliferation, oncogenesis	Hwang et al., 2017
Chronic myeloid leukemia	AIC-47 and imatinib/↓PBTP1/↓PKM2	Perturbation of energy metabolism	Shinohara et al., 2016
Gastric cancer	miR-133b/↓PTBP1	Negative regulation of the Warburg effect	Sugiyama et al., 2016
Oral squamous cell carcinoma	PTBP1/SRSF3	Overexpression of SRSF3, oncogenesis	Guo et al., 2015
Ovarian cancer	(1) PTBP1/CDC42 (2) ↓OVAAL/PTBP1/p27	(1) Filopodia formation, proliferation, invasiveness (2) Cellular senescence	He et al., 2015 ; Sang et al., 2018
Bladder cancer	(1) miR-145/↓KLF4/↓PTBP1/↓PKMs (2) PTBP1/MEIS2/PKM2	(1) Perturbation of the Warburg effect and inhibition of carcinogenesis (2) Lymph node metastasis, tumor stage, histological grade, and poor prognosis	Minami et al., 2017 ; Takai et al., 2017 ; Xie et al., 2019
Pancreatic ductal adenocarcinoma	(1) Gemcitabine/PTBP1/PKM2 (2) Linc-ROR/↓miR-124/PTBP1/PKM2	(1) Resistance to gemcitabine (2) Resistance to gemcitabine	Calabretta et al., 2016 ; Li et al., 2016
Breast cancer	(1) PTBP1/PKM2 (2) PTBP1/PEN-PI3K/Akt (3) PTBP1/FGFR1α/FGFR1β splicing	(1) Warburg effect, tumorigenesis, and transformation properties (2) Proliferation, invasion, and metastasis (3) Proliferation and migration	He et al., 2014 ; Ouyang et al., 2018 ; Zhao et al., 2019
Renal cell cancer	(1) PTBP1/PKM2 (2) ↓PTBP1/HIF-1α pathway	(1) Proliferation, migration, and invasion (2) Inhibit cell migration, invasion, and angiogenesis	Jiang et al., 2017 ; Shan et al., 2018
Glioblastoma	(1) ↓miR-124/PTBP1/ANXA7/EGFR (2) BAF45d/PTBP1 (3) PTBP1/USP5 (4) ↓CircSMARCA5/SRSF1/SRSF3/PTBP1	(1) Glioblastoma progression (2) Malignant phenotype and undifferentiated cellular state (3) Oncogenesis (4) Invasion and migration	Izaguirre et al., 2012 ; Ferrarese et al., 2014 ; Aldave et al., 2018 ; Barbagallo et al., 2018
Neuroblastoma	(1) MYCN/PTBP1/PKM2 (2) Hydrogen peroxide/PTBP1/sGC	(1) Proliferation, metastasis, and poor overall survival (2) Cellular adaptation to oxidative stress	Cote et al., 2012 ; Zhang SL et al., 2016
Glioma	(1) PTBP1/RTN4 (2) PTBP1/IRES-like element/ADAR1 p110	(1) Proliferation, invasion, and migration (2) Gliomagenesis and proliferation	Cheung et al., 2009 ; Yang et al., 2015
Colorectal cancer	(1) ↓miR-1 and ↓miR-133b/PTBP1/PKM2 (2) DDX6/c-Myc/PTBP1 (3) PTBP1/ATG10/EMT-associated proteins (4) PTBP1/cortactin	(1) Warburg effect (2) Warburg effect (3) EMT, migration and invasion (4) Migration and invasion	Taniguchi et al., 2015 , 2016 ; Jo et al., 2017 ; Wang et al., 2017
Colon cancer	(1) ELK1/MYC/PTBP1/RAS-MAPK (2) ↓TPBP1/TRAIL and ↓PTBP1/PKM2 → PKM1 (3) Apigenin/↓PTBP1/↓PKM2	(1) Tumorigenesis (2) Apoptosis and perturbation of the Warburg effect (3) Warburg effect and oncogenesis	Hollander et al., 2016 ; Kumazaki et al., 2016 ; Shan et al., 2017

Cancer, splicing and the EMT

In cancers of epithelial origin, which constitute the vast majority of cancers (Christofori 2006), the oncogenic process starts out as excessive cellular proliferation accompanied by angiogenesis in order to sustain the newly established tumour mass (Hanahan et Weinberg 2011). Cancers which are diagnosed in these early stages have the best clinical outcome (Whitaker 2020). The break between early stages and advanced stages of cancer is secondary to the invasion-metastatic cascade. The first step, invasion, sees epithelial cells from the tumour front acquire mesenchymal traits in order to delaminate, break the basement membrane and enter the surrounding conjunctive tissue. There, the second step can take place: intravasation. In this step, the cancer cells which had broken away from the initial tumour now find their way into the microvasculature of the vascular and lymphatic systems. These cells can then circulate around the body and are as such referred to as circulating tumour cells (CTCs). In order to establish a new tumour, these cells have to subsequently leave the circulatory system, in a step called extravasation, and colonise a new location. The CTCs then revert to an epithelial phenotype which can sustain rapid proliferation, and subsequently form a new tumour. In some cases, the cells which broke free from the initial tumour may never gain entry into the circulatory system, and may form localised secondary tumours (Valastyan et Weinberg 2011; Thiery 2002). **(Figure 22)**. Considering all this, we realise that understanding and gaining control of the first step of the metastatic cascade would be an incredible leap forward in preventing aggressive forms of cancer. So how exactly do cells change their phenotype, from epithelial to mesenchymal, and back again?

The epithelial to mesenchymal transition (EMT) in its broadest terms can be characterised by a loss of adherence between an epithelial cell and its partners, with a concomitant loss of its apico-basal polarity and instead gain of a front-back polarity **(Figure 23)**. From a mechanistic point of view, this necessitates the loss of cellular junction proteins such as claudins and occludins. The canonical EMT associated cellular junction protein is E-cadherin, whose downregulation has long been used as a marker of EMT. In view of this, the transcription factors associated with EMT induction have been classified based on their direct or indirect repression of the E-cadherin gene *CDH1*. On one hand, SNAI1, SNAI2, ZEB1, ZEB2, KLF8 and E47 directly repress *CDH1*. On the other hand,

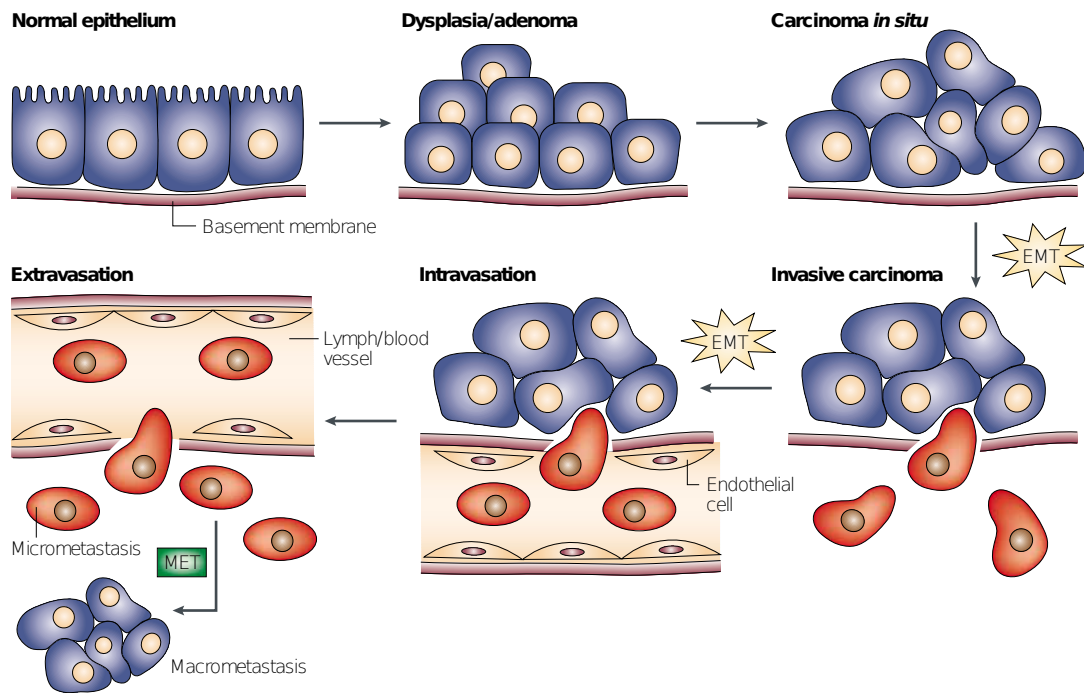


Figure. 22. The sequential steps of the metastatic cascade

Epithelial sheets undergo initial abnormal proliferation and primary tumour formation. The epithelial to mesenchymal transition promotes the initial invasion of the underlining tissues and of the microvasculature. Cells can then disseminate in the body before before exiting the circulation and forming secondary tumours while reverting to a more epithelial phenotype. (from Thiery et al. 2002)

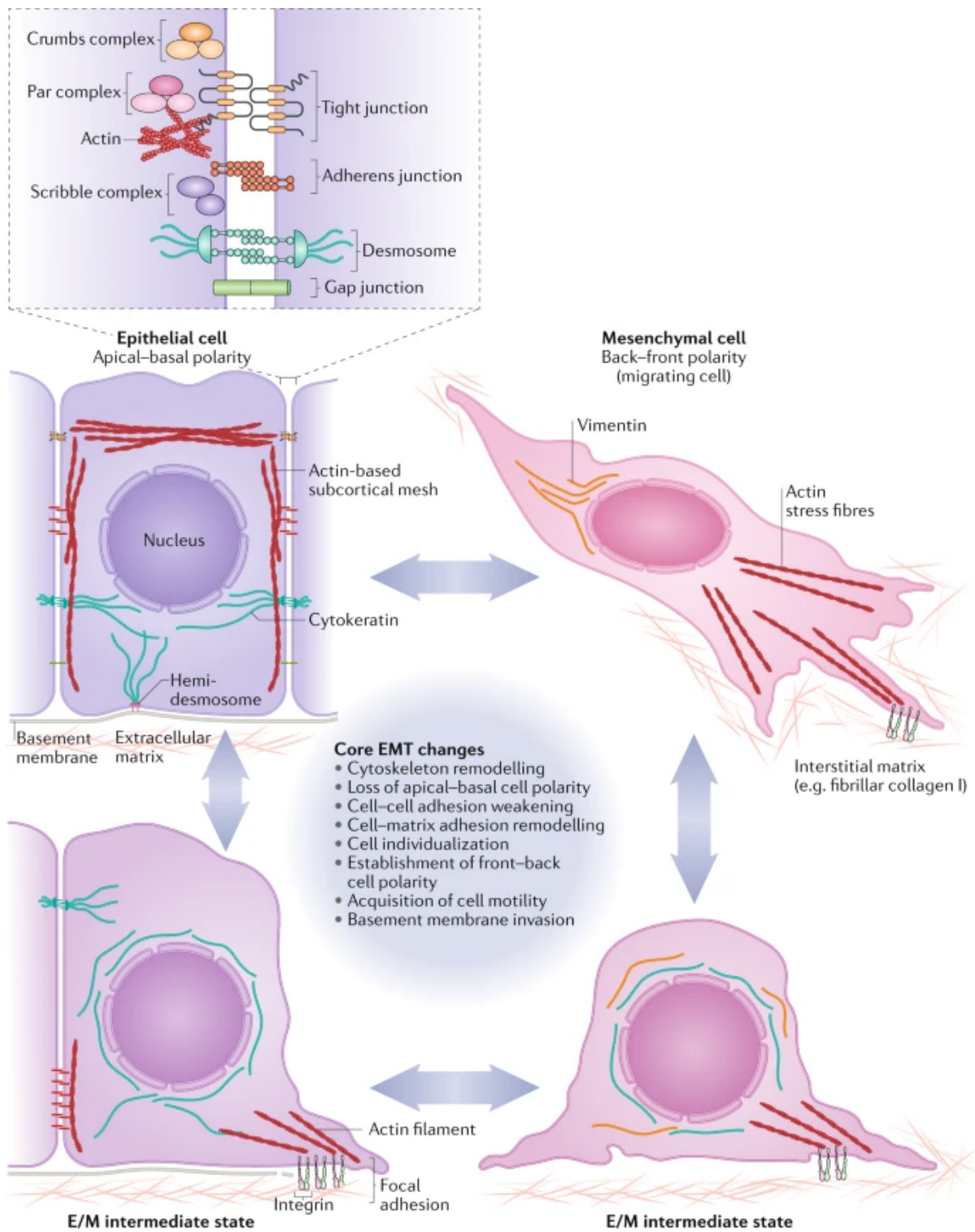


Figure. 23: The EMT as defined by the EMT international association (from Yang et al. 2020)

GSC, TCF4, FOXC2, PRR1 and TWIST are indirect repressors of *CDH1* (Peinado, Olmeda, et Cano 2007; J. Yang et Weinberg 2008). Changes in expression and activity of these classical EMT transcription factors of course lead to more consequences than simply E-cadherin loss, and there are therefore many molecular events which have been used as an EMT readout, the most frequently used being expression of Vimentin, Fibronectin and N-Cadherin (Zeisberg et Neilson 2009). Furthermore, these transcription factors are capable of regulating each other both transcriptionally and post-transcriptionally.

During embryogenesis, the EMT happens first during gastrulation when the epiblast cells of the primitive ectoderm differentiate into primary mesenchymal cells. Later on during development, EMT is also crucial for the correct formation of the secondary palate, which ensures the separation of the oral and nasal cavities of mammals (Nawshad, LaGamba, et Hay 2004; Martínez-Alvarez et al. 2004; Takigawa et Shiota 2004). In adults, the EMT also occurs physiologically during wound healing, and pathologically during cancer as described above (Kalluri 2009). There is however quite some contrast between the EMT which occurs during development and that which occurs during the metastatic cascade. In the former, cells differentiate fully from epithelial to mesenchymal states, whereas in the latter, epithelial cells tend to gain some mesenchymal traits while retaining most of their original properties. This is referred to as partial-EMT (P-EMT), and is thought to be the main mechanism involved during metastasis, perhaps even allowing migration of whole groups of cells (Pal et al. 2021a; Grigore et al. 2016; Saxena, Jolly, et Balamurugan 2020). The co-expression of both mesenchymal and epithelial markers is said to give cells an advantage in surviving in and exiting the vascular system (M. Yu et al. 2013). With the advent of single cell sequencing, markers expressed only in subsets of cells undergoing partial EMT, which would otherwise have been lost in a bulk assessment, are coming to light, and provide researchers with a spectrum of markers corresponding to the spectrum of cellular states (Pal et al. 2021a). In HNSCC, and by using single cell analysis of intra-tumoral heterogeneity, P-EMT genes have been identified which correlate with poor patient prognosis, despite no overall expression of EMT transcription factors (Puram et al. 2017a). Canonical EMT transcription have also been associated with poor prognosis in HNSCC (Wan et al. 2020) which highlights both the heterogeneity of the disease and the necessity to take both EMT and P-EMT into consideration when assessing the potential aggressivity of a tumour.

In such a plastic cellular landscape, besides transcription factor activity, post-transcriptional regulation is thought to be a strong component of the regulation of EMT (Nieto et al. 2016). This potentially places the fine tuning potential of alternative splicing at the forefront of P-EMT regulation. In line with this, RNA binding proteins are frequently up or down regulated in cancer, and mutations in RBPs associate with abnormal splicing events of which many are cancer drivers or other cancer hallmarks (Sebestyén et al. 2016). In one study of ZEB1-induced EMT in human non-small-cell lung cancer cells, ESRPs and RBM47 were reported to work in cooperation towards an epithelial splicing pattern, while QKI promoted a mesenchymal pattern (Y. Yang et al. 2016). In another study, this time in breast cancer cells induced into EMT by TWIST activation, ESRP1 was once again found associated with EMT relevant splicing events, as well as PTBP1 and RBFOX2 (Shapiro et al. 2011a).

AIMS OF THE STUDY

The many examples of the involvement of p63's isoforms during development, in the maintenance of adult tissues, and in oncogenic mechanisms highlight the pleiotropic functions of this transcription factor. The sometimes overlapping, sometimes mutually exclusive, and sometimes opposing effects of its isoforms underscore their requirement for tight regulation. A large part of the balance of p63 isoforms in a cell comes from post-transcriptional regulation and more precisely from control of its alternative splicing. If some examples of control of TP63 mRNA stability have been described, so far absolutely no information exists regarding the mechanisms controlling TP63 isoforms production. My thesis project therefore aimed to explore the regulation of TP63's isoforms at the RNA level, and investigate the functional impacts of modified isoform ratios in a human epithelial context.

The first axis of the project revolves around the discovery of a novel regulatory factor controlling the choice of alternative splice sites which operate the switch between producing TP63 γ or the α , β and δ isoforms. We identified the RNA binding protein PTBP1 as a negative regulator of the production of the TP63 γ isoform and demonstrated its direct repression of TP63 γ isoform production. The work that went into this aspect of the project has been summarised as the article "The splicing factor PTBP1 represses TP63 γ isoform production in squamous cell carcinoma" and is deposited as a preprint on www.biorxiv.org (<https://biorxiv.org/cgi/content/short/2021.10.01.462767v1>). This work is currently being submitted for publication.

Considering the published evidence towards differential roles of p63 α and p63 γ in epithelial cell biology, the second axis of my work was an attempt at identifying broad phenotypic shifts or more specific molecular events associated with a modification of the balance of TP63 isoforms in HaCaT keratinocytes or in HNSCC cell lines. To this end, we looked for modifications in the expression of epithelial or mesenchymal markers in cells with modified ratios of TP63 isoforms. We also investigated effects on cellular migration and proliferation, and we measured the expression of previously described TP63 target genes after overexpression or depletion of specific isoforms. Considering the exploratory

nature of this work, most presented data for this part was not as thoroughly replicated as it was for the first part.

At the protein level, detection of p63' minority isoforms γ and β is complex due to low expression levels and lack of specific antibodies. It is further complexified by proteolysis of p63 α that leads to accumulation of lighter proteins that are in the same molecular weight range as *TP63* γ . This means that immunoprecipitation and identification of the native proteins is tricky, if not outright impossible without isoform-specific antibodies. To try and solve this problem, we collaborated with the Etablissement Français du Sang (EFS) to try and produce novel monoclonal mouse antibodies which could either recognise all *TP63* isoforms, or specifically recognise the Pp3 α or p63 γ isoforms.

Finally, since *TP63* circular RNAs have been identified as part of an oncogenic axis in lung squamous cell carcinoma, we sought out to determine whether they are also expressed in our P63 positive HNSCC cell lines. Furthermore, these circRNAs include regions which we have described as regulated by PTBP1. We therefore enquired upon the possibility that PTBP1 also regulates *TP63* circRNA biogenesis.

RESULTS

Chapter 1. Functional characterisation of the involvement of the RNABP PTBP1 in the regulation of *TP63* isoform production.

1.1. Article: “PTBP1 controls *TP63* alternative splicing in HNSCC tumour cells”

TITLE PAGE

THE SPLICING FACTOR PTBP1 REPRESSES *TP63* γ ISOFORM PRODUCTION IN SQUAMOUS CELL CARCINOMA

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keywords : Alternative splicing, RNA binding protein, RNA targets, EMT, biomarkers, p53 family

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ABSTRACT

The *TP63* gene encodes the transcription factor p63. It is frequently amplified or overexpressed in squamous cell carcinomas. Owing to alternative splicing, p63 has multiple isoforms called α , β , γ and δ . The regulatory functions of p63 may be isoform-specific. The α isoform inhibits the epithelial to mesenchymal transition (EMT) and controls apoptosis, while the γ isoform promotes EMT. Here, we observed in TCGA data that a high ratio of the *TP63 γ* isoform to the other isoforms is a pejorative factor for the survival of patients with head and neck squamous cell carcinoma (HNSCC). We therefore addressed the regulation of the γ isoform. In several tissues (GTEx data), the expression of the RNA-binding protein PTBP1 (polypyrimidine tract binding protein 1) is negatively correlated with the abundance of *TP63 γ* . Accordingly, we demonstrated that PTBP1 depletion in HNSCC cell lines leads to an increase in abundance of the γ isoform. By RNA immunoprecipitation and in vitro interaction assays, we showed that PTBP1 directly binds to *TP63* pre-mRNA in close proximity to the *TP63 γ* -specific exon. The region around the *TP63 γ* -specific exon was sufficient to elicit a PTBP1-dependent regulation of alternative splicing in a splice reporter minigene assay. Finally, we demonstrated that the regulation of *TP63 γ* production by PTBP1 is conserved in amphibians, revealing that it encounters a strong evolutionary pressure. Together, these results identify *TP63 γ* as a prognostic marker in HNSCC, and identify PTBP1 as a direct negative regulator of its production.

INTRODUCTION

The *TP63* gene encodes a conserved transcription factor, p63, controlling epithelial development and homeostasis (1,2). In Humans, heterozygous mutations in *TP63* lead to developmental syndromes affecting ectodermal tissue derivatives (3). Overexpression or amplification of *TP63* is observed in multiple cancer types generally from epithelial origin (4) and p63 staining therefore serves as a classification biomarker in some malignancies including skin cancer (5). It discriminates carcinoma from non carcinoma breast cancer types, and lung carcinoma from lung adenocarcinoma in combination with other markers (6,7). It is also a prognostic marker as *TP63* loss is associated with metastatic progression in head and neck squamous cell carcinoma (8).

TP63 encodes multiple protein isoforms owing to alternative promoters and alternative splicing or polyadenylation. The *TP63* gene structure is evolutionary conserved and beside exons, several intronic regions also present similarities among vertebrates. The complexity of the *TP63* gene structure allows for the production of multiple protein isoforms (**Fig. 1A**). This conserved complexity suggests some evolutionary pressure presumably associated with the function of the different isoforms (9,10).

The first promoter generates mRNAs encoding TAp63 isoforms comprising the N-terminal transactivation (TA) domain. The mRNAs produced from the second promoter encode only a partial TA domain and the encoded proteins are the Δ Np63 isoforms. The Δ Np63 isoforms are the most abundant ones in epithelial tissues, where they are present mainly in basal cells. They carry most of the p63 functions required for epidermal proliferation and differentiation. The TA isoforms are preferentially expressed during female germline differentiation where they protect the genome from accumulating DNA damage in a way similar to p53 in somatic cells (11). A tissue-specific 5' TA variant coined GTA TP63 has been described in Hominid testis where it is proposed to similarly act as a guardian of the male germ line (12).

Several C-terminal ends are produced from combinations of cassette and alternative terminal exons. These isoforms encode p63 proteins harboring different combinations of the C-terminal domains. The longest isoform p63 α contains a phosphodegron (PD) (13), a sterile-alpha-motif (SAM) (14), a transcriptional inhibitory domain (15) and a regulatory sumoylation region (16). The shortest mRNA isoform TP63 γ encodes only the oligomerisation and DNA binding domains. It is generated by the use of an internal terminal exon and thus has a 3' untranslated region (3'UTR) different from the other C-terminal isoforms. Since all p63 isoforms contain the oligomerisation domain, they may all interfere with each other's functions. Most described functions of the α isoform concern its involvement in epithelia, and the p63 γ isoform is unable to support proper

epithelial development (17). Instead, p63 γ promotes the onset of an epithelial to mesenchymal (EMT) transition in keratinocytes (18), promotes osteoblastic differentiation (Curtis et al. 2015) and favors terminal myogenesis (20). The functions of p63 are therefore strongly dependent on the regulation of the production of the different 5' and 3' isoforms in a tissue-specific manner. Despite the acknowledged importance of the differential role of the p63 C-terminal isoforms, the regulation of the multiple alternative splicing events involved in p63 pre-mRNA maturation remains unaddressed (21).

Regulation of alternative splicing events requires the assembly of the spliceosome machinery onto splice sites (noted 5'SS and 3'SS with respect to the introns) and the definition of the cleavage and polyadenylation sites (CPA) (22). The tissue-specific choices among different alternative splicing and polyadenylation events are generally controlled by a combination of regulatory sequence elements, silencers or enhancers and their cognate trans-acting factors, generally RNA binding proteins (RBPs). The differential tissue distribution of these RBPs will often account for the tissue-specific regulation of alternative splicing (23). We reasoned that by analyzing relative abundance of RBPs and *TP63* alternative splicing events in RNASeq data from many tissues we could prioritize the RBPs most likely to intervene in TP63 splicing.

Here, we show by combining exploration of TCGA and GTEX Data, RNA/protein biochemistry and reverse genetics experiments that higher inclusion of the γ exon is associated with poorer survival of Head and Neck squamous Cell Carcinoma (HNSCC) patients and we identify the RBP PTBP1 as a direct inhibitor of the inclusion of the γ exon in HNSC cell lines. This regulation of TP63 alternative splicing by PTBP1 appears to be essential as it is evolutionarily conserved, being present in *Xenopus laevis*, a model amphibian that diverged from amniotes about 360 Million years ago. Targeting the PTBP1-mediated regulation of *TP63* expression may therefore be a means to modify TP63 isoform ratio.

MATERIAL AND METHODS

Cell lines

HNSC cell lines (TCP-1012) were obtained from ATCC, HaCaT cells were obtained from CLS Cell Lines Service. HeLa, HaCaT, FaDu and Detroit 562 cells were cultured in DMEM medium (Gibco), A253 cells were cultured in Mc Coy's 5A (Gibco), SCC-9 and SCC-25 cells were cultured in DMEM/F12 (Gibco) supplemented with 400 ng/mL of hydroxycortisone (H0888, Sigma-Aldrich). For siRNA depletion, cells were seeded on 6-well plates at 150000 cells per well. After seeding, a jetPRIME transfection mix (Polyplus) was added, containing 100 pmol of target siRNA or negative controls.

Xenopus embryos microinjection

Xenopus laevis eggs were obtained from WT females and fertilized using standard procedures (24). When indicated 30 ng of MoPtbp1 (25) or 30 ng of control morpholino (GeneTools) was injected into each blastomere of two-cell embryos in a volume of 13.8 nl, using a Nanoject II (Drummond). For rescue experiments, 1 fmol of mRNA-encoding morpholino-resistant PTBP1-V5R was co-injected. Embryos were allowed to develop at 22 °C and collected at stages 26 according to Nieuwkoop and Faber stages (26). Total RNA was extracted using RNeasy columns (Qiagen) from a pool of 3 embryos at stage 26. RNA were analysed by RT-PCR for 25 cycle using radiolabeled PCR primer.

Minigene construction and analysis

The TP63 exon 12 minigene was constructed by gibson assembly of Beta-globin and TP63 gene fragments obtained by PCR amplification from HaCaT genomic DNA using oligonucleotides pairs hGbE1_E2, hTP63_gamma, hGb_E3 and cloned into ApaI, NheI digested pmirglo (Promega). Construction was controlled by sequencing. HaCaT cells were transfected with plasmid minigene and selected using G418 (Gibco). Expression and splicing of the minigene was assessed by RT-qPCR using primers presented in the reagent table.

RNA extraction, reverse transcription and qPCR

Total RNA was extracted from cells using nucleospin RNA kits (Macherey Nagel, #740955.50) following the manufacturer's instructions. One µg of total RNA was reverse transcribed with superscript II enzyme (Invitrogen) and random primers (Invitrogen #58875). Dilutions of cDNA (1:20) were amplified with PowerSybr Green Master mix (Appliedbiosystems, #4367659) using a Quantstudio (TM) 7 flex real-time PCR 384 well system.

Western-blot

Cells were washed with PBS 1X and lysed in 2X Laemmli sample buffer (4% SDS, 20% glycerol, 0,125M tris-Hcl pH 6.8, 2.5% β-mercaptoethanol, 0.2% bromophenol blue). Denatured

protein samples (10', 95°C) were loaded onto 8-12% SDS-PAGE gels. Transfer onto nitrocellulose membranes (Amersham Protran 0.45µm NC) was performed using the trans-blot turbo transfer system (Biorad). Membranes were blocked for one hour with a 5% fat-free milk TBST solution (1X TBS, 0.1% Tween 20), incubated with primary antibody solutions (16h, 4°C). After three washes in TBST (5'), the membranes were incubated with anti-mouse or anti-rabbit secondary antibodies coupled to HRP (1h at 20°C), washed three times in TBST (5'). Revelation was performed on an Amersham AI680 imager with either ECL-select (Amersham) or West pico PLUS (Thermo-fisher scientific) chemiluminescent substrate, depending on anticipated signal strength.

TCGA data analysis.

TP63 isoform and splicing in HNSC patient samples were quantified using clinical and expression data from firebrowse.org and splicing quantification from TCGA splice SEQ (27). All expression, splicing and survival analysis were conducted in R. For kaplan-meier estimation, the package survival was used and a log rank test (28) applied. Scripts are available from Gitlab (project ID:29285922).

GTEX data analysis

To identify RBPs with tissue expression correlated to TP63 tissue specific junction uage, we analysed RNASeq data from GTEx Analysis 8.0. The GTEx data are composed of 17 382 samples from 54 different tissues obtained from 980 donors. For each sample s , the total number of reads T_s is calculated. The median read depth Mrd is calculated form each sample and a read depth normalisation factor (N_s) for each sample as:

$$N_s = \frac{T_s}{Mrd}$$

Normalized gene expression Egs for gene g and sample s is computed as:

$$Egs = \sum_{i=1}^n junction_{gsi} \times N_s$$

Tissue and samples expressing TP63 are selected and used for correlation analysis. RBPDB (29) provided a list of RBPs for which we calculated the RNA expression in the TP63 expressing samples. For each of the 21 TP63 junctions the junction usage is computed as:

$$junctionusage_{is} = \frac{junction_{is}}{\sum_{i=1}^{21} junction_{is}}$$

The pearson correlation coefficient and associated p-value is calculated for each RBP with the junction usage of the TP63 junction. Top correlated RBPs are then selected for further analysis. R scripts are available on gitlab (project ID:29285922).

Fluorescent RNA

Transcription templates were synthesized by PCR (GOTAQ G2, Promega) using a hybrid T7promoter/sequence-specific forward primer and a sequence-specific reverse primer. PCR products were checked on native agarose gel and purified on columns (DC5, Zymoresearch). Fluorescent RNAs were transcribed (37°C, 3h) in (Transc. buffer (1X), 5mM dTT, rNTPs 0.5 mM each, Cy3-UTP 0.1mM (Jena Bioscience), RNAsin (1U/μl, Promega), T7 RNA polymerase (1,25 U/μl), DNA template (12,5 ng/μl)). RNA were purified on G50 sephadex (GE-healthcare). and controlled by denaturing electrophoresis and fluorescent detection (Typhoon FLA 9500). Cy3 fluorescence and RNA absorbance were quantified on a De Novix DS-11 spectro/fluorimeter.

Protein production and EMSA

The sequence encoding hsPTBP1 (NM_002819.5) was obtained from sourcebiosciences (IRAU969B052D). The PTBP1 ORF was PCR amplified and subcloned by gibson cloning into pET21A+ (Novagen) digested by *XhoI* and *NheI*. The 6xHis-tagged hsPTBP1 was expressed in *E.Coli* (BL21) after induction by IPTG (1mM), and purified on nickel column using standard procedures. Protein was eluted with 250 mM Imidazole and concentrated on vivaspin (30KDa cutoff). Purified protein was resuspended in (Sodium Cacodylate pH7.0 20mM, NaCl 100mM, EDTA 0.5 mM, DTT 1mM).

The EMSA experiment were performed by mixing dilutions of hsPTBP1 in RNA Binding buffer (RBB: Sodium Cacodylate pH7.0 10mM, BSA 0.1μg/μl, yeast tRNA 0.1μg/μl, NaCl 100mM, MgCl₂ 1mM, DTT 1mM, Rnasin (Promega) 0.4U/μl, Heparin (Sigma) 1U/μl) with an equal volume of labeled RNA in RBB. RNA protein complexes were analyzed on native polyacrylamide gels (30). Bound and free RNA were quantified on a Typhoon FLA 9500. To estimate the K_d, a non linear model of the form (bound/total RNA) ~ 1/(1 + K_d/[PTBP1]) was fitted to the data for each RNA using R script (31).

PTBP1 complex immunoprecipitation

Ten millions cells were lysed in (50mM TrisHCl, 0.1M NaCl, 1 % NP40, 0.1 % SDS, 0.5 % Deoxycholate , protease inhibitor cocktail P8340 0.1 %, RNAsin 400U, TurboDNase 10U, DTT 1mM) and incubated 10 min at 37°C. Prior to immunoprecipitation, lysates were partially digested with RNaseT1 (Ambion) (0.25U per mg of lysate 5 min, 37°C). Lysates were pre-cleared with 50μl of dynabeads Protein-G magnetic for 1 hr at 4°C. The cleared supernatants were incubated with 50 μl of beads pre-coated with 40 μg of anti-PTBP1 antibodies (clone BB7) or mouse IgG Isotype Control (Invitrogen). Immunoprecipitations were conducted overnight at 4°C with constant shaking, then washed successively with IP300 (50 mM HEPES-K pH 7.5, 300 mM KCl, 0.05% NP-40, 0.5 mM DTT), IP500 (50 mM HEPES-K pH 7.5, 500 mM KCl, 0.05% NP-40, 0.5 mM DTT),

IP750 (50 mM HEPES-K pH 7.5, 750 mM KCl, 0.05% NP-40, 0.5 mM DTT) and finally once with WB (20mM Tris-HCl pH 7.4; 10mM MgCl₂; 0.2%Tween20).

One tenth of the immunoprecipitated samples were used for analysis of immunoprecipitated proteins by SDS–PAGE and western blotting. Co-immunoprecipitated RNAs were isolated by proteinase K treatment (30 min, 37°C in 150 µl of PK buffer (50 mM Tris pH 7.4, 1M NaCl, 0.5% NP40, 0.5mM EDTA, 0.1% SDS, 2M Urea, 1% Deoxycholate) containing 50 µg tRNA and 12 U proteinase K (ThermoFisher) followed by phenol–chloroform extraction and ethanol precipitation. RNAs were analyzed by RT–PCR or RT-qPCR .

RESULTS

Higher TP63 γ exon inclusion is associated with poor outcome in HNSC patients.

While many HNSC tumors overexpress *TP63* (**Fig. 1B** and (Campbell et al. 2018)), the expression and impact of its C-terminal isoforms on patient survival is unclear. We used TCGASpliceSeq data (27) to evaluate inclusion of exons composing the splice variants of *TP63* in HNSC patients. The $\Delta NTP63$ isoforms are characterized by transcription initiation at exon 4 (**Fig.1A and SFig. 1A**) and account for more than 99 % of total *TP63* (**Fig.1C**) in both normal and tumor samples. The *TATP63* isoforms are barely detectable. Among the two terminal exons 12 and 16, the inclusion of exon 16 accounts for about 98 % of the *TP63* RNA (isoforms α , β or δ , see **SFig.1B** for isoform structure). Conversely, usage of exon 12, found exclusively in *TP63 γ* , is weak (around 2 % in average). A small but significant increase in the usage of exon 12 is observed in tumors samples compared to normal tissues ($p=0.010$, Wilcoxon test) with a parallel decrease in exon 16 (**Fig.1C**). The exclusion of exon 15 measures *TP63 β* , while the exclusion of the pair of exons 14 and 15 measures *TP63 δ* . No difference could be observed between tumors and normal sample for these splicing events. We sought to determine whether differential exon usage in tumors could be associated with difference in survival probability for patients (**Fig.1D**). The measure of inclusion of exon 12 or of both exon 14+15 in tumor samples could discriminate patient survival rates. Higher inclusion ($\text{psi} \geq 4.07\%$) of the exon 12 was associated with a lower survival of patients (median half-life = 862 days compared to 2319 days, $p = 0.00363$). Lower inclusion of exon 14+15 was also associated with a more moderate decreased survival ($\text{psi} < 98.5\%$, median half-life = 1466 days compared to 2319 days, $p = 0.0177$). A higher inclusion of *TP63 γ* exon appears therefore detrimental for patient survival.

Candidate RBPs controlling TP63 γ exon inclusion.

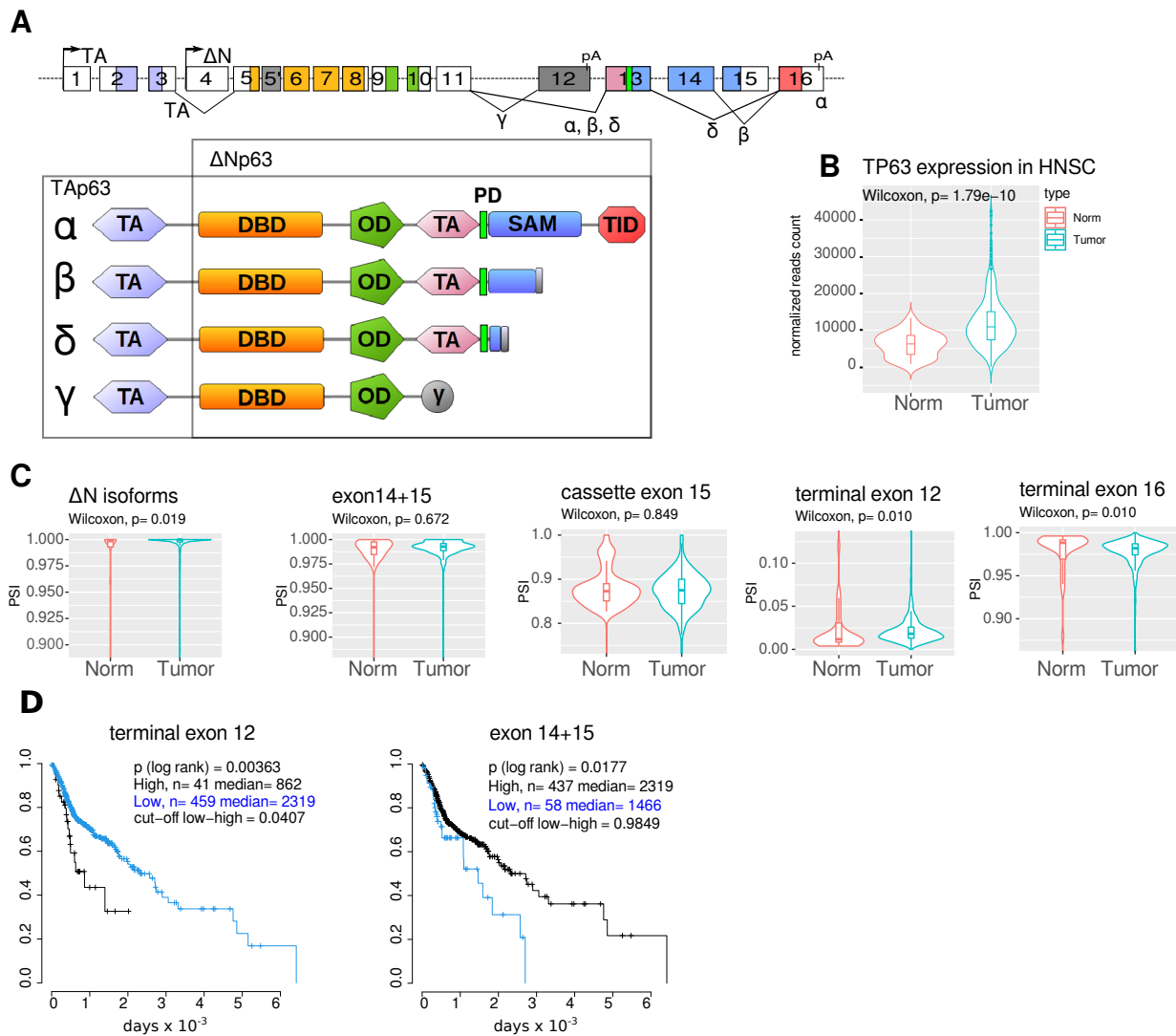


Figure 1: TP63 splicing from HNSC patients

A) TP63 exon/intron structure (upper scheme) and corresponding encoded protein isoforms with peptidic domains colour- encoded. TA, ΔN denote the promoters. Exons are numbered according to GTEX. Alternatively spliced junctions and cleavage and polyadenylation events are indicated with solid lines joining exons and pA respectively. Terminal exon 16 is present in isoforms α, β or δ, and terminal exon 12 in isoform γ. The cassette exons 14 and 15 are present in isoform α while the isoform β lacks exon 15 and the isoform δ lacks both. The domains are colored according to the exon/intron structure. TA-Transactivation domain, DBD-DNA Binding Domain, OD-Oligomerisation Domain, SAM-Sterile Alpha Motif, TID-Transactivation Inhibitory Domain, PD-PhosphoDegron. B) Gene-wise expression of TP63 in normal and tumor samples from HNSC patients. C) Quantification of TP63 splicing by measuring inclusion of specific exons (percent spliced-in, PSI) in normal and tumor samples. Differential expression between normal and tumor samples was assessed using Wilcoxon rank sum test D) Patients were segregated based on the high or low inclusion of exon 12 (threshold = 4.07%) or exon 14+15 (threshold = 98.49 %) in tumor samples. Patient survival was assessed on a Kaplan-Meier graph in the two classes and statistical differences appraised by a log rank test.

Because tumors samples may be highly heterogeneous due to tumor type, location or mingled tissues, we sought to identify potential regulators of *TP63* splicing using normal tissues data. We searched for RNA-binding proteins (RBPs) whose expression correlated to *TP63* junction usages. RNASeq junction quantification were obtained from the GTEX consortium (33). They represent quantifications from 54 different tissues or conditions. The correspondence between GTEX and TCGA nomenclatures for *TP63* is shown in SF1A. We calculated the normalized expression for *TP63* in each sample and all tissues. Among the 54 tissues, ten (10/54, 18.5 %) were selected as expressing *TP63* (**Fig.2A**). These tissues where mainly of epithelial origin, as expected for *TP63*, but EBV-transformed lymphocytes and skeletal muscles also showed a notable *TP63* expression.

For each tissues and sample we quantified the usage of the 21 individual *TP63* junctions (**SFig.2A**). The junction usage is strikingly different between tissues (**SFig.2B**). We can distinguish three groups of tissues: Muscle, Epithelia, and EBV-transformed lymphocytes. Eleven junctions are used at similar levels across tissues. Among those, four are very weakly used (J4-6, J5-5', J5'-6 and J13-16) and seven are constitutive splicing events included at high levels in all tissues. Ten junctions are differentially used across tissues. Junctions (J1-2, J2-3, J3-5, J4-5) are representative of differential promoter usage between epithelial tissue (ΔN isoforms favored) and muscle and EBV-transformed lymphocytes (TA isoforms favored). Muscle specific differences (J11-12 and J11-13, red boxes) are visible. Theses differences are not observed in EBV-transformed lymphocytes. The competition between these two mutually exclusive splicing reactions (J11-12, J11-13) sharing an identical 5' splice site allows for the production of the γ or [α , β , δ] terminal isoforms. The quantification of these two reactions (**Fig.2B**) clearly shows that they are oppositely regulated, with epithelial tissues favoring the exclusion of the exon γ and the inclusion of the γ terminal exon being promoted in skeletal muscle.

We hypothesized that correlations between RBP expression and junction usage could allow us to pinpoint RBPs acting as splicing enhancers or splicing silencers affecting the tissue specific inclusion of the γ exon. Using the RBPs represented in the RNA Binding Protein Database (29), we first determined their expression in *TP63* expressing tissues. We could analyse the expression levels of 386 different RBPs in the different tissues (**Fig.2C**). Based on individual samples, we computed the correlations between RBP expression and junction usage relevant to the γ exon (J11-12 and J11-13). Among the 10 most correlated RNA binding proteins (**Fig.2D**), eight were positively correlated and two were negatively correlated to γ exon inclusion (J11-12). As expected the correlation was inverted when considering the alternative junction (J11-13). These 10 RBPs are

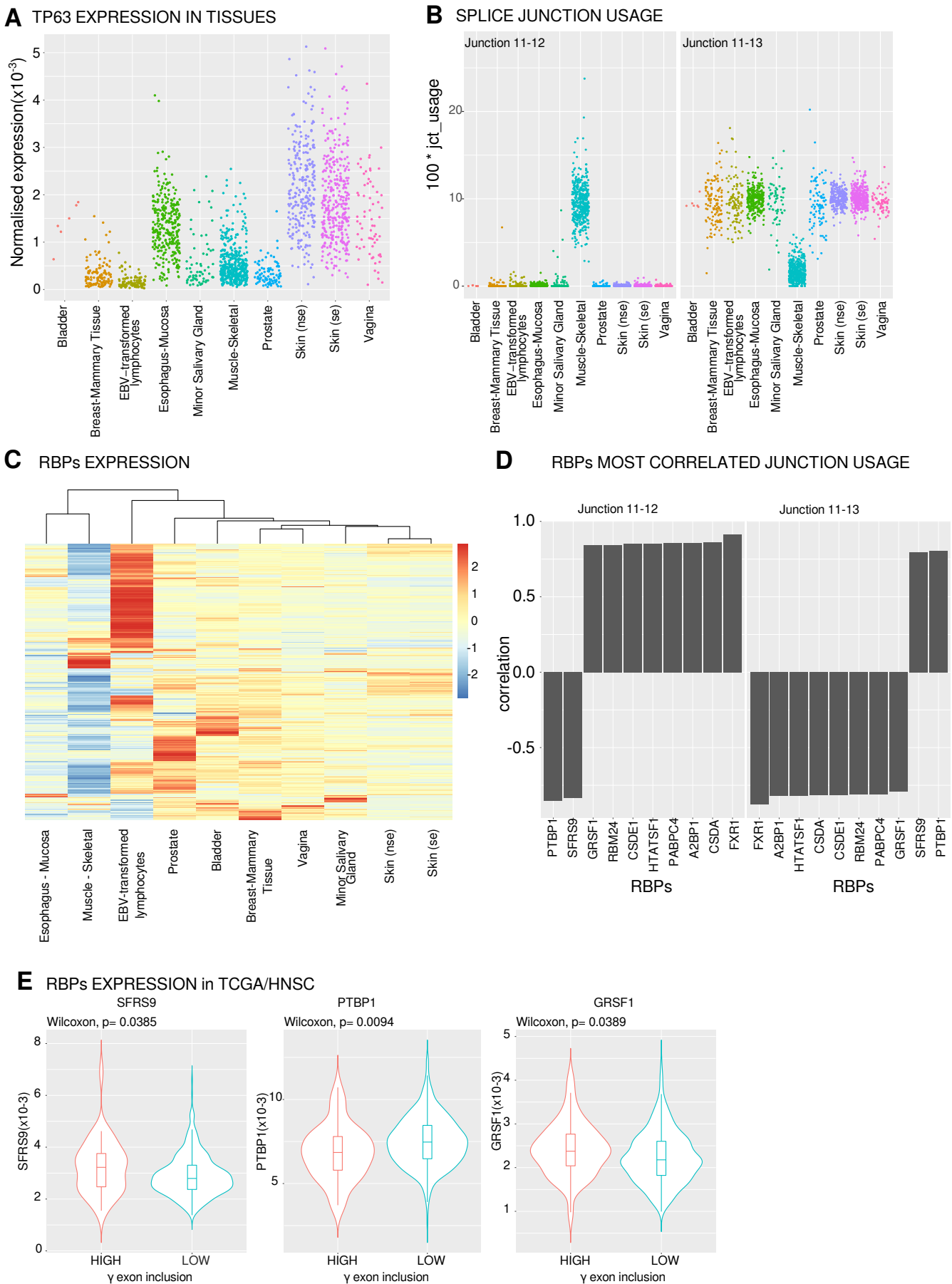


Figure 2: Identification of PTBP1 as a RBP correlated to TP63 γ terminal exon splicing.
 A) TP63 gene expression in samples from TP63 expressing tissues (normalized reads count) (se, nse, (non-) sun exposed). B) Quantification of the usage of junctions 11-12 and 11-13 pertinent to γ terminal exon inclusion. C) RBP expression heatmap and hierarchical clustering of tissues based on RBP expression from GTEX data. D) Top 10 RBPs most correlated to the junctions involved in γ exon inclusion. E) Expression of SFRS9, PTBP1 and GRSF1 in two classes of patients with high or low γ exon 12 inclusion (threshold =4.07%) in tumors.

strongly expressed in muscle (FXR1, GRSF1, RBM24, CSDE1/UNR, HTATSF1, PABPC4, A2BP1/RBFOX1, CSDA/YBX3) or in epithelial tissues (PTBP1, SRSF9).

To determine whether expression of the selected RBPs was coherent with higher γ exon inclusion in HNSC patients with poor survival, we compared the differential expression of the RBPs in tumors selected for having high or low levels of γ exon that discriminate among patient survival (**Fig.1C**). Of the 10 RBPs tested, only three (SFRS9, PTBP1, GRSF1) are differentially (Wilcoxon, $p < 0.05$) expressed at the RNA level between tumors from patients with high or low γ exon inclusion (**Fig.2E**). *SFRS9* is more highly expressed in samples with high γ exon inclusion (Wilcoxon, $p = 0.0385$), which is not in accordance with the GTEX data where *SRSF9* is negatively correlated to γ exon inclusion (**Fig.2D**). *PTBP1* and *GRSF1* are low and high, respectively, in patients with high gamma (Wilcoxon, $p = 0.0094$ and $p = 0.0389$, **Fig.2E**). The same RBPs are negatively and positively correlated, respectively, with γ in GTEX data (**Fig.2D**). Therefore *PTBP1* and *GRSF1* can coherently be involved in γ exon regulation. However, because *GRSF1* is mainly a mitochondrial RBP and is weakly expressed in epithelial cells (34) while *PTBP1* is a well defined alternative splicing regulator expressed in epithelial tissues, we chose to test whether experimental depletion of *PTBP1* could enhance *TP63* γ exon inclusion in a panel of HNSCC cell lines (FaDu, SCC9, SCC25, Detroit562 and A253) from different tissues of origin.

PTBP1 represses endogenous TP63 γ exon inclusion in squamous carcinoma cell lines.

Using two different antibodies we analyzed p63 and p63 α -specific expression by western-blot (**Fig.3A**) in a panel of HNSC cell lines. Both antibodies detected a major isoform around 70 kDa, coherent with the major Δ NP63 α isoform (MW 66 kDa). In addition to the 70 KDa protein the pan-p63 antibody (4A4) detected lower molecular weight bands undetected using the α -specific antibody (D2K8X). This suggested that isoforms other than Δ NP63 α are expressed at lower levels in the tested cells. The identity of these isoforms can not be inferred from these experiments. We used RT-qPCR to evaluate the abundance at the RNA level of total *TP63* and of its three predominant isoforms (α , β , γ). We estimated that global *TP63* expression levels were higher in SCC25, then A253 and SCC9, then FaDu, Detroit 562 and non transformed keratinocytes HaCaT. As expected, it is barely detectable in HeLa cells used as a negative control (**Fig. 3B**). We used isoform-specific RT-qPCR to quantify C-terminal isoforms (**Fig.3C**). In all cell types tested, the α isoform is the major one ranging from 87 to 95 % of *TP63* mRNA. Detroit 562 cells have the lowest α proportion. Accordingly, β and γ isoforms are in higher proportions in Detroit 562 cells with 8 % and 2.7 % of each respectively. The expression of the β and γ isoforms are variable among the cell lines with the Detroit 562 and the SCC25 cells having the highest relative proportion of

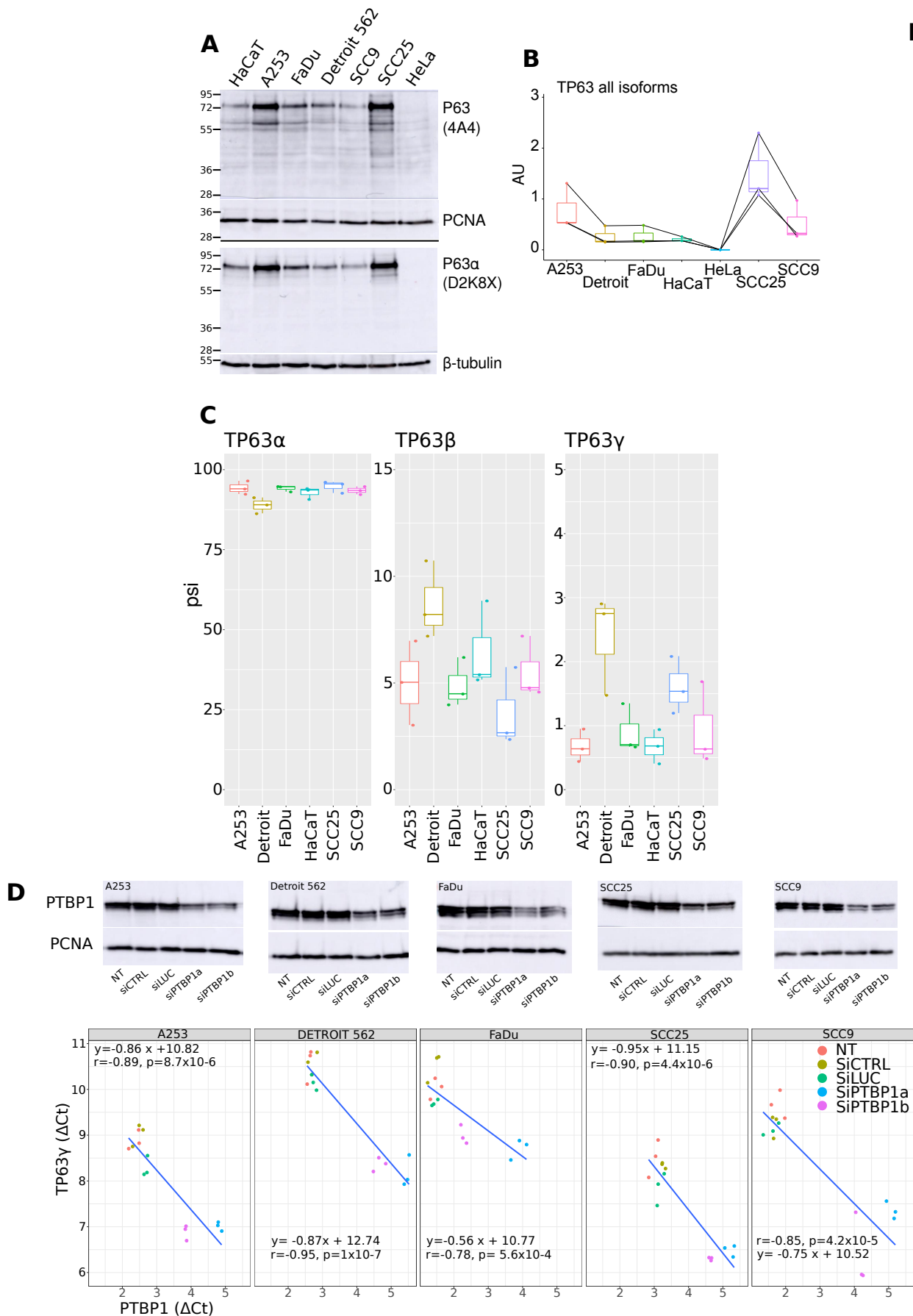


Figure 3: PTBP1 controls TP63 splicing in HNSCC cell lines.

A) p63 expression was evaluated in HaCaT, HeLa and HNSC cells by western blot and immunodetection using a pan-p63 antibody (4A4) or a p63α-specific antibody (D2K8X) (n=3). Even loading was controlled using PCNA or β-tubulin antibodies as indicated. B) TP63 RNA expression was measured by RT-QPCR with one pair of primer detecting all isoforms and quantification was normalised to a calibration curve obtained from TP63 plasmid DNA dilutions (n=3). C) Quantification of TP63α, β or γ percent spliced-in using isoform-specific primer pairs (n=3). D) upper panel, quantification of TP63γ isoform and PTBP1 RNA in control and PTBP1 depleted HNSC cell lines (n=3). Lower panel, evaluation of PTBP1 depletion by western blot (n=3), PCNA serves as a loading control.

TP63 γ (2.75 and 1.5% respectively) while other cell lines had all less than 1 % of TP63 γ mRNA (**Fig.3C**).

We efficiently depleted PTBP1 in the five HNSC cell lines using two different siRNAs (**Fig.3D**, upper panels). In all five cell lines, depletion of PTBP1 led to an increase in TP63 γ abundance as shown by the lower TP63 γ Δ CT in PTBP1 depleted cells (**Fig.3D**, lower panels). The TP63 γ levels were therefore inversely related to PTBP1 levels. This demonstrates that PTBP1 represses the accumulation of the TP63 γ isoform in HNSC cell lines.

PTBP1 binds to TP63 pre-mRNAs sequences in vivo on the γ exon 3'Splice site and CPAs.

Direct regulation of TP63 splicing would require binding of PTBP1 to regulatory elements located in the pre-mRNA of interest. To determine whether PTBP1 was bound to TP63 pre-mRNAs *in vivo* we immuno-precipitated specifically PTBP1/RNA complexes from HaCaT extracts and analyzed the pre-mRNA content of the complexes by RT-PCR and RT-qPCR (**Fig.4A, 4B**). Presence of TP63 pre-mRNA was specifically detected in input and PTBP1 immunoprecipitates but not using control Ig or beads only. To determine whether PTBP1 was preferentially associated to TP63 pre-mRNA or mRNA, we used primers specific to TP63 mRNAs (ex8-ex9, ex11-12) or pre-mRNA (int10-ex11, int11-ex12, int12-ex13). As shown in **Fig.4B**, the most significant and robust enrichment was observed with the primer pair specific to the pre-mRNA region closest to the γ exon (int11-ex12). Control RT-qPCR performed in absence of reverse transcriptase did not show PCR amplification (data not shown). Therefore, on TP63 pre-mRNA, PTBP1 is preferentially bound to the region that is up-regulated following PTBP1 depletion.

The RBPmap database details known binding data for hundred of RBPs (35). Binding motifs for PTBP1 represent predicted binding sites (PBS) and were plotted along the TP63 γ exonic region (**Fig.4C**). Examination of the genomic sequences surrounding TP63 γ exon identifies 4 blocks of conservation among vertebrates : an upstream intronic region (a), a region overlapping the 3' splice site (b), a broader area in the γ 3'UTR (c) and a smaller region overlapping the cleavage and polyadenylation (CPA) site (d).

We performed electrophoretic mobility shift assay (EMSA) using recombinant PTBP1 and fluorescently labelled RNA molecules to determine the affinity of PTBP1 for RNA sequences representing the conserved sequence elements (**Fig.4D** and **SFig.3**). We quantified the fluorescent RNAs in bound and unbound PTBP1 complexes and calculated a Kd based on PTBP1 concentration (31). As presented in **Fig.4D**, RNA fragment 1 (FRAGT 1) composed solely of β -globin RNA sequences, FRAGT2 that is composed in part of β -globin RNA sequences and in part of the TP63 intronic region, FRAGT 5, FRAGT7 located totally in the γ exon, and CPA1 located close to the

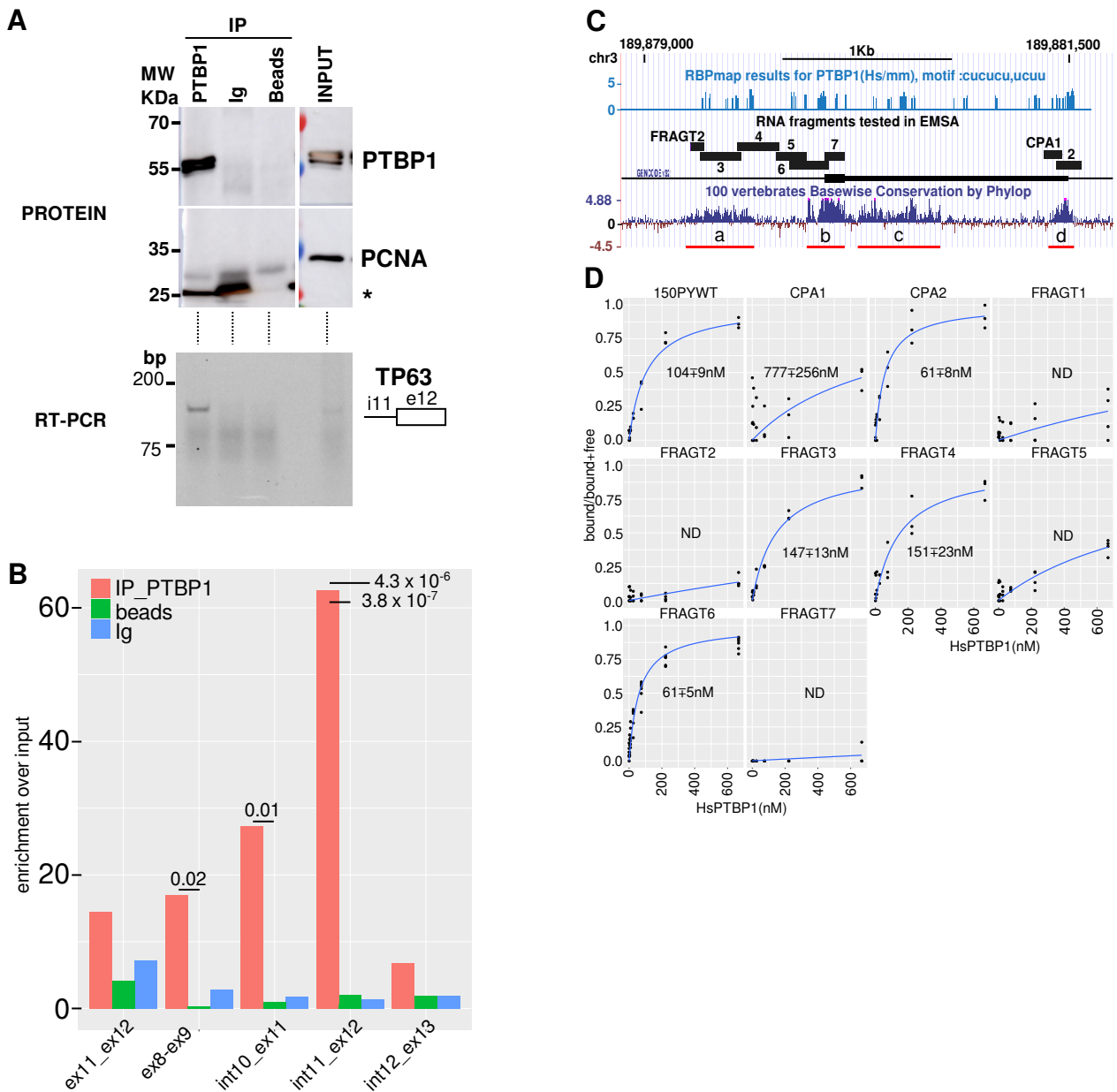


Figure 4: PTBP1 binds to TP63 premRNAs sequences.

A) Immuno-purification of PTBP1/RNA complexes or control experiments (Ig, beads only) from HaCaT cell extracts. Immunopurification was controlled by PTBP1 and PCNA western-blot as indicated in input (10%) or IP samples (upper panels). Protein MW are indicated on the side of the membranes. The asterisk denotes the light-chain of the IgG. Lower panel, RT-PCR detection of TP63 pre-mRNA with primers located in intron 11 and exon 12 in the indicated samples. B) RT-qPCR detection of different mRNA or pre-mRNA portions of TP63 from IP or control samples relative to input signal. Statistical assessment between PTBP1 and control IP was measured by a student-test ($n=3$). C) TP63 exon 12 genomic locus and position of the RNAs tested in RNA/protein shift assays. Upper lane, RBPmap predicted PTBP1 binding sites. Middle lane, positions and names of the RNA fragment tested along the TP63 exon 12 genomic sequence. Lower lane, nucleotidic conservation among vertebrates with regions of high conservation labeled a,b,c,d as discussed in the text. D) PTBP1/RNAs binding curves (bound/ bound + free) obtained for the indicated RNAs ($n \geq 4$). The calculated Kd and their s.d. is shown for each plot.

cleavage and polyadenylation site, remain unbound to PTBP1. FRAGT3, FRAGT4 and FRAGT6 and CPA2 associate strongly with human PTBP1 protein with binding affinities better than (CPA2, FRAGT6, ~ 60 nM) or comparable to (FRAGT3, FRAGT4, ~ 150 nM) the positive PTBP1 binding control RNA (150PYWT, ~ 104 nM) (36).

Altogether, this demonstrated the direct association of PTBP1 on regulatory regions surrounding the γ exon 3'Splice site and CPAs on the TP63 pre-mRNA *in vivo* in an epithelial cell lines.

PTBP1 represses TP63 γ exon inclusion in a minigene in epithelial cells.

If PTBP1 can control TP63 γ exon inclusion by directly binding to these cis-elements, then the splicing of a minigene composed of the TP63 genomic sequence should be under the control of PTBP1. To test this, we stably transfected HaCaT cells with minigenes containing TP63 genomic sequences within an efficient β -globin splicing context. The structure of the minigene is shown in **Fig.5A**. We depleted PTBP1 in HaCaT keratinocytes using either of two different siRNAs (siP1A and siP1B, **Fig.5B**). As previously shown in HNSC cell lines (**Fig.3D**), PTBP1 depletion strongly increases the abundance of TP63 γ in HaCaT cells (**Fig.5C**). This demonstrates that, like in transformed cells, the accumulation of the endogenous TP63 γ isoform is at least partly repressed by PTBP1 in non-transformed HaCaT keratinocytes.

The TP63 minigene splicing is also dependent on PTBP1 abundance (**Fig.5D**). Upon PTBP1 depletion, the splicing between E2 and the γ terminal exon was increased 2 times (paired t.test, pvalue = 0.0016, n=3) while the alternative possibility, splicing of E2 to E3, was decreased about 2 times (paired t.test, pvalue = 0.004, n=3). This demonstrates that the genomic region surrounding the γ terminal exon is sufficient to allow for its PTBP1-dependent regulation.

PTBP1 represses TP63 γ inclusion since at least 350 Millions years.

Clusters of PTBP1 PBS are located in-between evolutionarily conserved regions a and b and in regions b, c and d (**Fig.4C**). Because of this interesting conservation of non coding regions, we addressed the regulation of the PTBP1-dependent regulation of the γ exon in an evolutionary distant organism. It is estimated that amphibians and mammals diverged from their common tetrapod ancestor roughly 360 millions years ago (37). Within amphibians, *Xenopus laevis* is a well-established model of vertebrate development. The exon/intron structure of the TP63 locus is well conserved between *Xenopus* and human with conserved coding exons (in blue on **Fig.6A**) serving as milestones along the sequence. In *Xenopus*, two annotated additional exons (11' and *, in green) are present (**Fig.6A**). Exon * is very weakly used and exon 11' encode an alternative peptide in

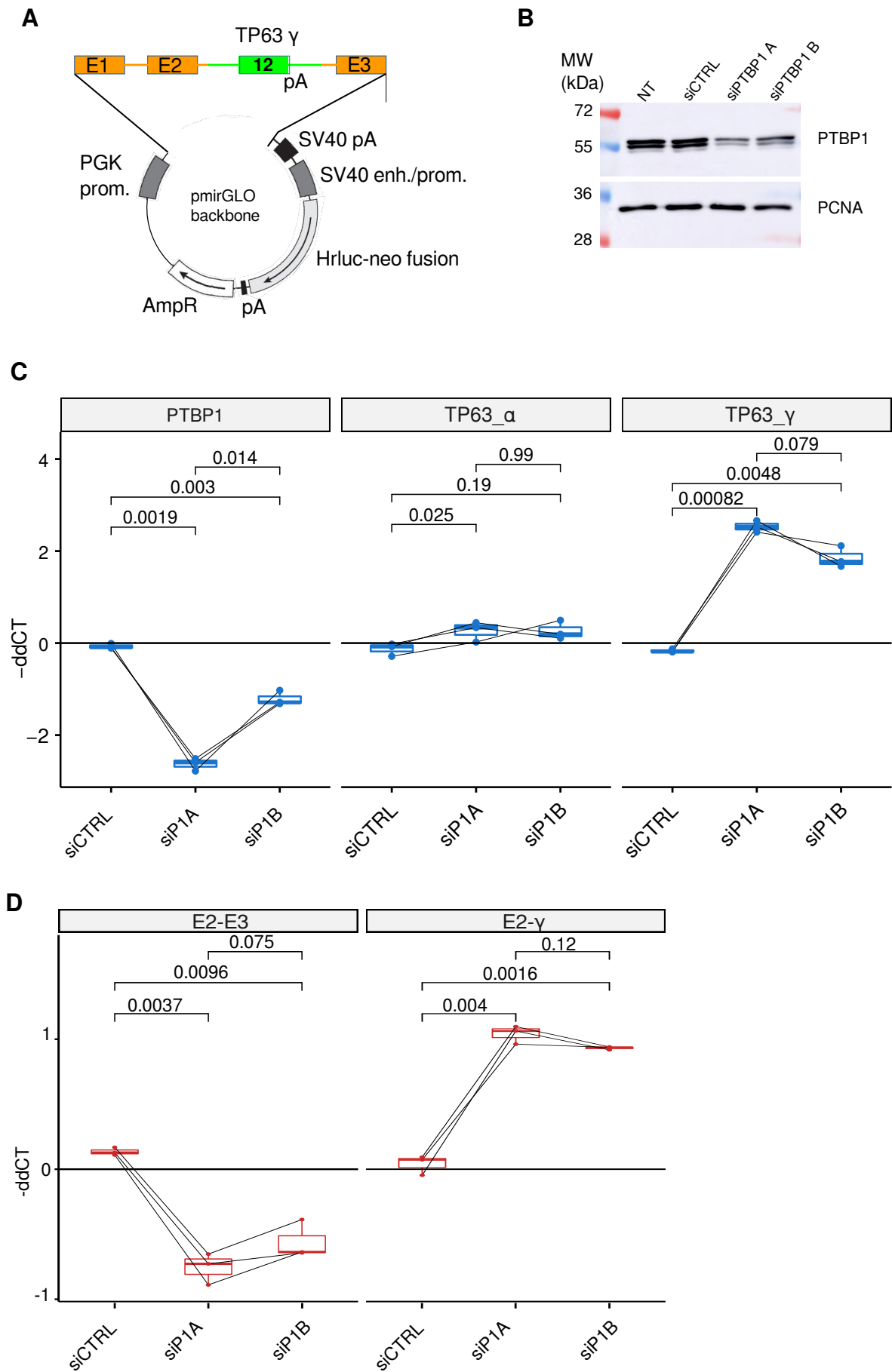


Figure 5: PTBP1 represses endogenous TP63 γ exon inclusion in HaCaT cells and on a TP63 γ exon minigene.

A) Structure of the TP63 minigene stably transfected in HaCaT cells. B-globin gene sequence are shown in orange, TP63 gene sequences are shown in green. B) Detection of PTBP1 in HaCaT cells after PTBP1 depletion mediated by two different siRNA (n=3). C) RT-qPCR quantification of PTBP1 levels and endogenous TP63 α and γ isoforms after siRNA-mediated PTBP1 depletion (n=3, t-test). D) RT-qPCR quantification of the RNA isoforms produced from the TP63 minigene (n=3, t test).

frame with the rest of the coding sequence. A terminal exon corresponding to the human gamma exon is present in both species.

Previously, we used antisense morpholino oligonucleotides to deplete Ptbp1 from developing embryos, and we analyzed RNA expression by RNAseq (25). Here, we re-investigated these data to focus on *Xenopus tp63*. Notably, the depletion of Ptbp1 led to an increase in the γ exon (12) inclusion in embryos as judged by the Sashimi plot on **Fig.6B**. On this example, reads spanning junction 11-12 or junction 11'-12 increased from 0 to 35, and from 5 to 56 respectively. This was accompanied by an accumulation of sequencing reads on the γ exon. To independently confirm these data, we analyzed *Xenopus* embryos by RT-PCR (**Fig.6C**). The analyzed embryos were previously injected with a control antisense morpholino oligonucleotide (lane 2), a morpholino against PTBP1 (lane 3), the same morpholino co-injected with an mRNA encoding PTBP1 made immune to the morpholino by a few nucleotidic substitutions (lane 4), or the rescue RNA alone (lane 5). As shown on **Fig.6C**, depletion of PTBP1 leads to the specific accumulation of RT-PCR products 11-12 and 11-11'-12, revealing an increased inclusion of the γ exon. That this increase is due to a specific change in RNA splicing in this region is shown by the unchanged signal when another region of *tp63* mRNA is amplified (primers in exons 14 and 15). Upon restoration of PTBP1 expression from an injected rPTBP1 mRNA which is resistant to morpholino inhibition, the γ exon inclusion was restored to control levels.

These results demonstrate that the repression of γ exon usage by the RNA binding protein PTBP1 is a mechanism that emerged at least in tetrapods and has been conserved across 360 Millions years of evolution. Together, these data reveal that PTBP1 controls TP63 γ exon inclusion by binding to discrete and conserved regulatory elements located in this pre-mRNA region. The γ exon inclusion is hence restricted by the presence of PTBP1, decreasing the abundance of the TP63 γ isoform in normal and cancerous epithelial cell lines where PTBP1 is abundantly expressed.

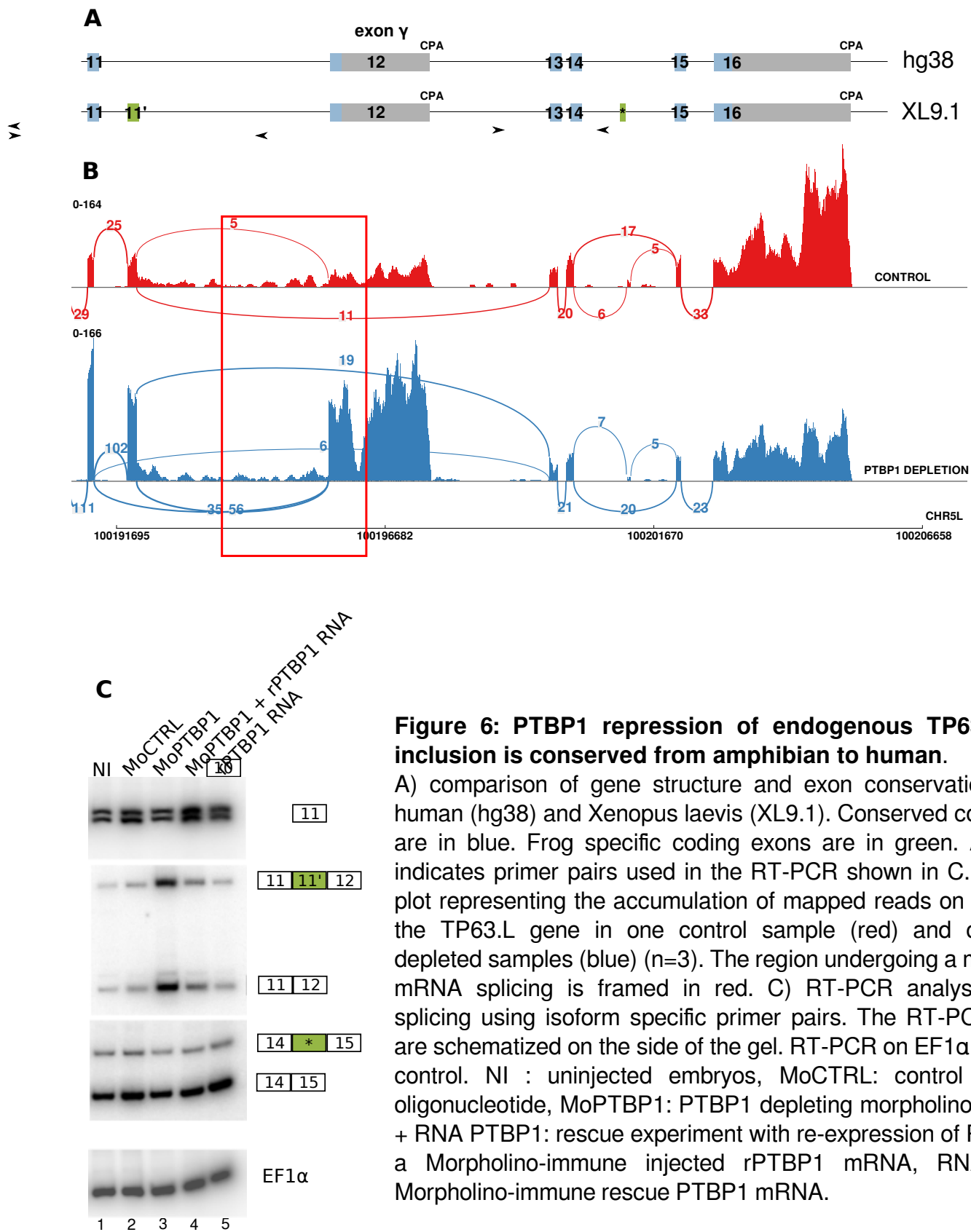


Figure 6: PTBP1 repression of endogenous TP63 γ exon inclusion is conserved from amphibian to human.

A) comparison of gene structure and exon conservation between human (hg38) and *Xenopus laevis* (XL9.1). Conserved coding regions are in blue. Frog specific coding exons are in green. Arrowheads indicate primer pairs used in the RT-PCR shown in C. B) Sashimi plot representing the accumulation of mapped reads on the locus of the TP63.L gene in one control sample (red) and one PTBP1 depleted samples (blue) (n=3). The region undergoing a major shift in mRNA splicing is framed in red. C) RT-PCR analysis of TP63 splicing using isoform specific primer pairs. The RT-PCR products are schematized on the side of the gel. RT-PCR on EF1 α serves as a control. NI : uninjected embryos, MoCTRL: control morpholino oligonucleotide, MoPTBP1: PTBP1 depleting morpholino, MoPTBP1 + RNA PTBP1: rescue experiment with re-expression of PTBP1 from a Morpholino-immune injected rPTBP1 mRNA, RNA rPTBP1: Morpholino-immune rescue PTBP1 mRNA.

DISCUSSION

Alternative splicing misregulation in cancer cells is emerging as a major theme in the alteration of gene expression observed during the cancerous process. This offer new routes of tumor classification or treatment (38). Using a heuristic approach we identified *PTBP1* as a major repressor of *TP63 γ* production in normal and malignant epithelial cells. This is the first identification of a trans-acting factors controlling the splicing pattern of *TP63* in a tissue-specific manner.

Predicted PTBP1 binding sites were located in evolutionarily conserved regions framing exon 12 in the *TP63* pre-mRNA and we demonstrated that PTBP1 binds with high affinity to discrete elements of the *TP63* pre-mRNAs *in vitro* and in cells. These elements coincide with the 3' SS and the CPA of the γ exon.

Model of γ terminal exon regulation by PTBP1.

PTBP1 is generally observed as an inhibitor of the splicing or polyadenylation reaction. However some cases of exon activation by PTBP1 have been reported (39). This inhibition is generally the consequence of the inhibition of the recognition of the 3' splice site by U2AF (40) or of the CPA site by CSTF (Castelo-Branco et al. 2004). On *Xenopus* tropomyosin mRNA, PTBP1 inhibits the function of an intronic silencer element located upstream of a terminal exon (42). The multiple RNA-recognition motifs of PTBP1 and its possible multimerisation on its targets offer multiple modes of interaction (43). PTBP1 binding to a pre-mRNA at several sites can either hinder the regulated exon or exclude it by creating a loop through interaction of PTBP1 in the upstream 5' and downstream 3' region surrounding the regulated exon (44). Gruber and colleagues (45) identified repeats of CU dinucleotides, reminiscent of PTBP1 binding sites, as preferentially associated with differentially used CPA in tumors versus normal samples of glioblastomas. *PTBP1* levels were strongly anti-correlated with the length of these RNAs suggesting that PTBP1 acts as an inhibitor of CPA on tandem UTRs harboring sequential polyadenylation sites. Binding of PTBP1 on the CU-rich regions around the CPA would hinder its recognition by the CPA-bound machinery favoring the alternative unmasked CPA. The context of the *TP63* γ exon is different as it is an internal terminal exon in competition with the splicing of a classic exon defined by 5' and 3'SS. As interactions between splicing factors involved in the recognition of the 3'SS and the CPA bound factors are central in the efficient processing of terminal exon (46), we can propose that by its dual binding to the 3'SS and the CPA, PTBP1 is likely interfering with the ability of U2AF and CPA factors to interact with 3'SS and CPA respectively and therefore impede their ability to define the γ exon.

In HNSCC cells depleted in PTBP1, the inclusion of the γ exon is still modest (see **Fig.3D**). It is therefore possible that additional inhibitory factors are at play in these cell lines. We can exclude that this limited effect is because of a redundant role of the orthologues of PTBP1 as co-depletion experiments of PTBP1 and PTBP2 barely increase the γ exon inclusion (data not shown). One alternative is that the limited γ exon inclusion in HNSC cells is due to intrinsic properties of this γ exon, with sub-efficient 3'SS and CPA that would only drive limited inclusion. In this case efficient terminal exon γ inclusion observed in other tissues would require specific factors to enhance exon γ inclusion. Such factors should be looked for in tissues where the exon γ is predominantly used and we can assume that germ cell or muscle cells may prove useful to identify such factors.

Which role for the γ terminal exon ?

The importance of TP63 in development is clearly demonstrated from works in numerous models. In *Xenopus*, *tp63* is required for the proper muco-ciliary epithelial development (47). The evolutionarily conserved regulation of the γ exon by PTBP1 suggests that the p63 γ harbors specific and required functions in certain cellular type or tissues. These functions may come from its impact on the p63 coding sequence, from a difference in the sequences of the 3'UTRs between γ and the α or β isoforms, or from more complex phenomenon such as regulation of circular RNA production as already described for the *TP63* gene (48). At the RNA level, because of its different 3'UTR, and the γ isoforms have a different susceptibility to miRNAs controlling TP63 expression than the α , β isoforms (49). It is therefore possible that the production of the γ isoform provides the cell with a way to escape some miRNA-mediated tissue-specific regulation of *TP63*. However this change in 3'UTR would also result in a change in the encoded p63 isoforms.

It is unlikely that the γ -specific peptide encoded in the terminal exon has any conserved functions by itself because of its important divergence between species. Indeed, only 15/37 (40.5%) aminoacids are conserved between *Xenopus* and Human γ peptides while the overall identity of Δ Np63 α proteins is 85 %. Both in Human and in *Xenopus*, the γ isoform is strikingly different from the α isoform. In Human, it is deprived of 231 amino acids forming the SAM domain, a transcription inhibitory domain, a transcription activation domain and a phosphodegron. These domains are of paramount importance for the correct function of TP63 as demonstrated by the Ankyloblepharon Ectodermal defects Cleft lip/palate (AEC) and Rapp-Hodgkin (RHS) syndromes associated with mutations altering these domains (50). Yet p63 γ retains the DNA binding and oligomerisation domains allowing the protein to form hetero-complexes that have altered transcriptional functionality (51).

In general, the ability of an alternative isoform to affect protein complex functions increases with the number of monomers composing the complex (52). p63 forms homo- or hetero-tetramers composed of various p63 or p73 proteins. It is therefore possible that even low amounts of the γ isoform affect the functions of p63 as a transcription factor. It is unclear yet if the low amount of TP63 γ at the RNA levels are strictly correlated to similarly low protein level. Indeed, expression experiments showed that Δ Np63 γ was the most stable p63 isoform in Hep3B cells (Petitjean et al. 2008). This may be due to Δ Np63 γ being deprived of the Fbw7 E3 ligase phosphodegron and of some of the lysine residues (K494,/505) involved in ubiquitylation and destabilisation of p63 α (13,53). Quantification of the γ isoform at the protein level is complex as no γ -specific antibodies are currently available. We may expect that state of the art mass-spectroscopy will allow for isoform specific quantification of protein in the near future (Lau et al. 2019).

The overexpression of Δ Np63 γ in the context of global p63 depletion leads to increased EMT in both a breast cancer cell line (55) and in a 3D organotypic model of invasion ((18). This p63 γ -associated EMT is promoted through a *TP63/SRC/SNAIL2* transcriptional axis (18). However in HNSC patients, we failed to observe any increased accumulation of SRC in TCGA tumor samples with higher expression of the gamma isoform (data not shown). Another possibility arises from the existing feed-back loop where Wnt/B-catenin signaling promotes *TP63* expression (47,56) and p63 represses Wnt/B-catenin signaling (57,58). This feedback loop could be essential for the maintenance of proper balance between differentiation and proliferation. One can speculate that this balance could be tilted by the modification of the activity of the p63 transcription factor associated with an altered ratio of the p63 C-terminal isoforms.

We observed that increased expression of *TP63* γ in tumors is associated with HNSC patients having a lower survival. While we can not conclude that this lower survival is a direct consequence of *TP63* γ expression, we identified PTBP1 as being more lowly in these tumor samples. It is therefore possible that the health status of these patients is associated with a PTBP1-specific altered splicing pattern that is reflected in the splicing of *TP63*. In this context measuring *TP63* isoforms in tumor biopsies could serve as a biomarker to identify patients with altered splicing patterns and potentially altered survival.

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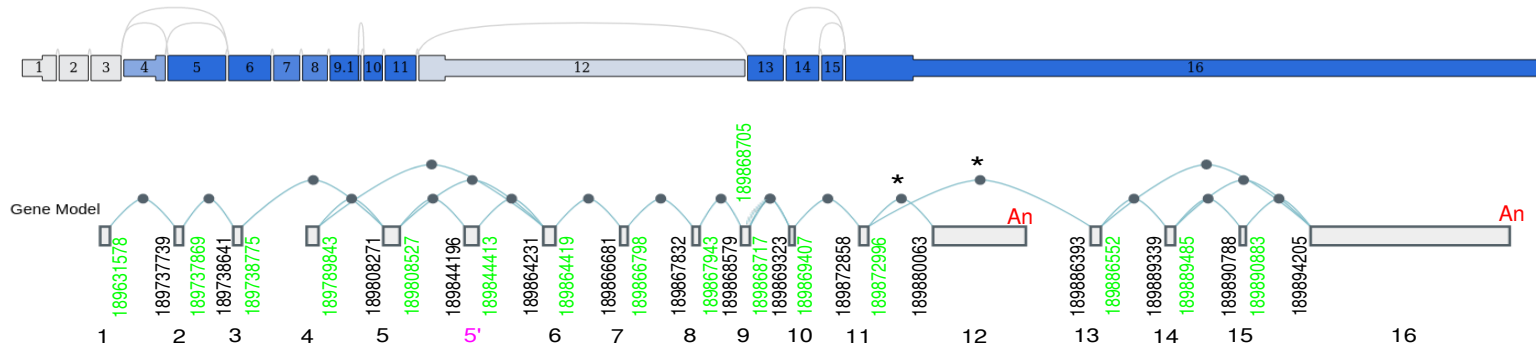
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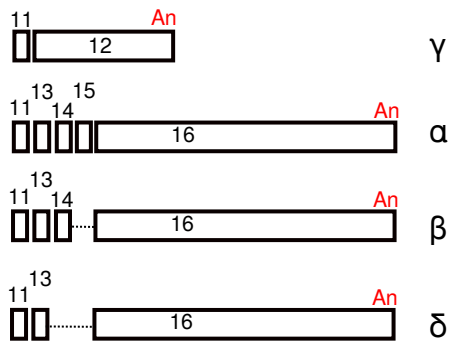
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A Correspondance between the TP63 gene model from TCGA and GTEX



B Exon composition of the different C-terminal isoforms

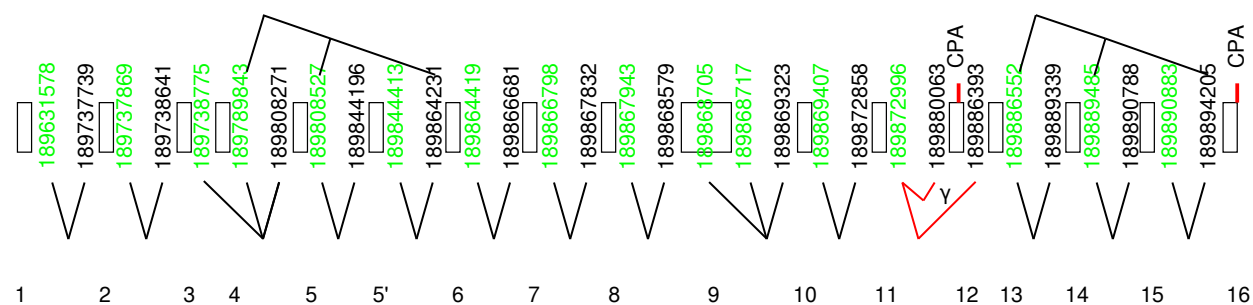


Supplementary Figure 1: TP63 gene structure

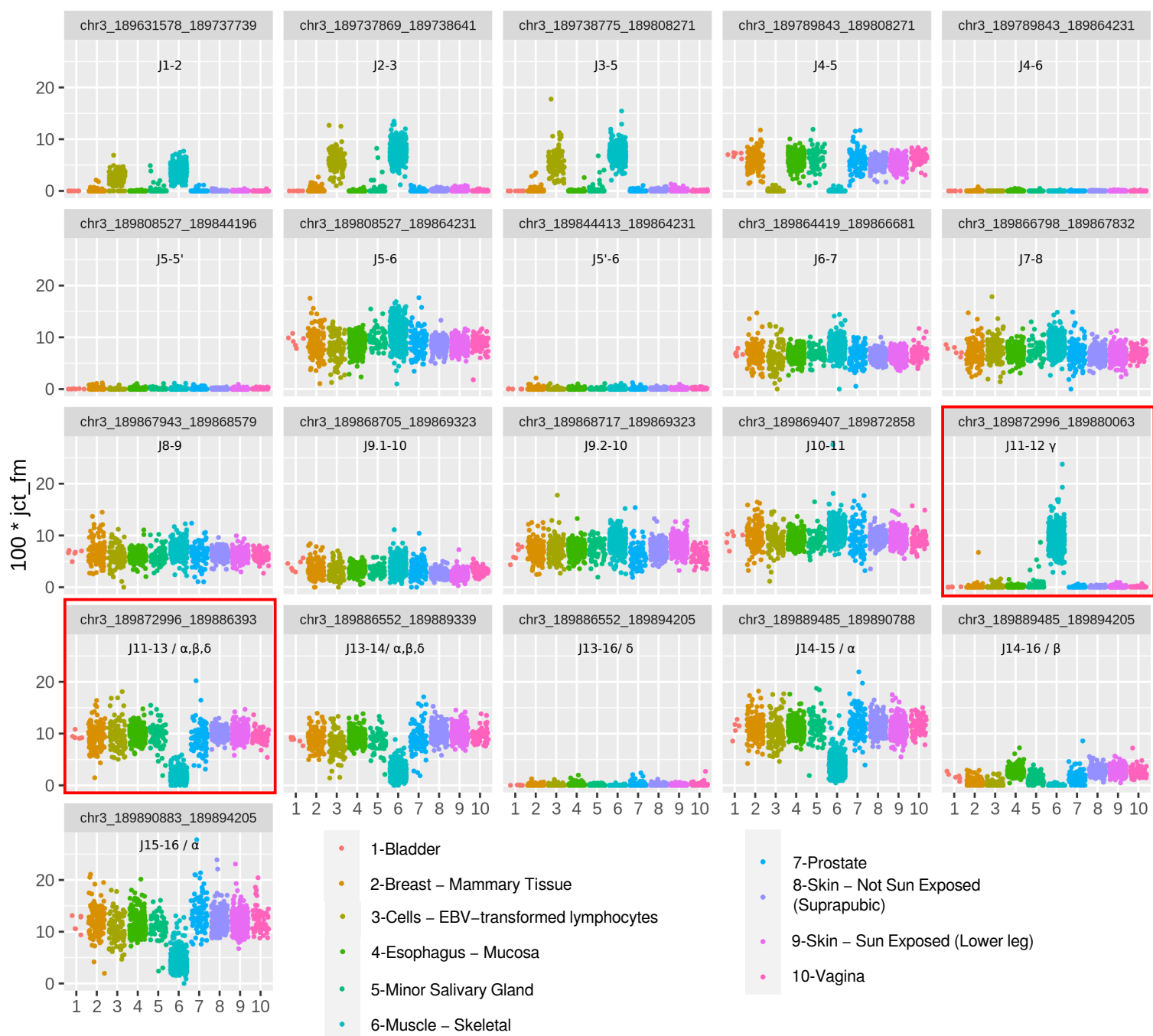
A) Correspondence of the TCGA (upper lane) and GTEX (lower lane) annotations for the TP63 locus. Hg38 chromosomal locations of 5' and 3' splice sites are shown in green and black respectively. The main difference between both annotations stands in the presence of an additional exon, noted 5' here, in the GTEX gene model. The exon numbering used throughout the manuscript is from the TCGA annotation and presented underneath the GTEX gene model. B) Exon composition of the C-terminal isoforms γ , α , β , δ .

SUPPLEMENTARY FIGURE 2

A TP63 gene structure - 21 junctions



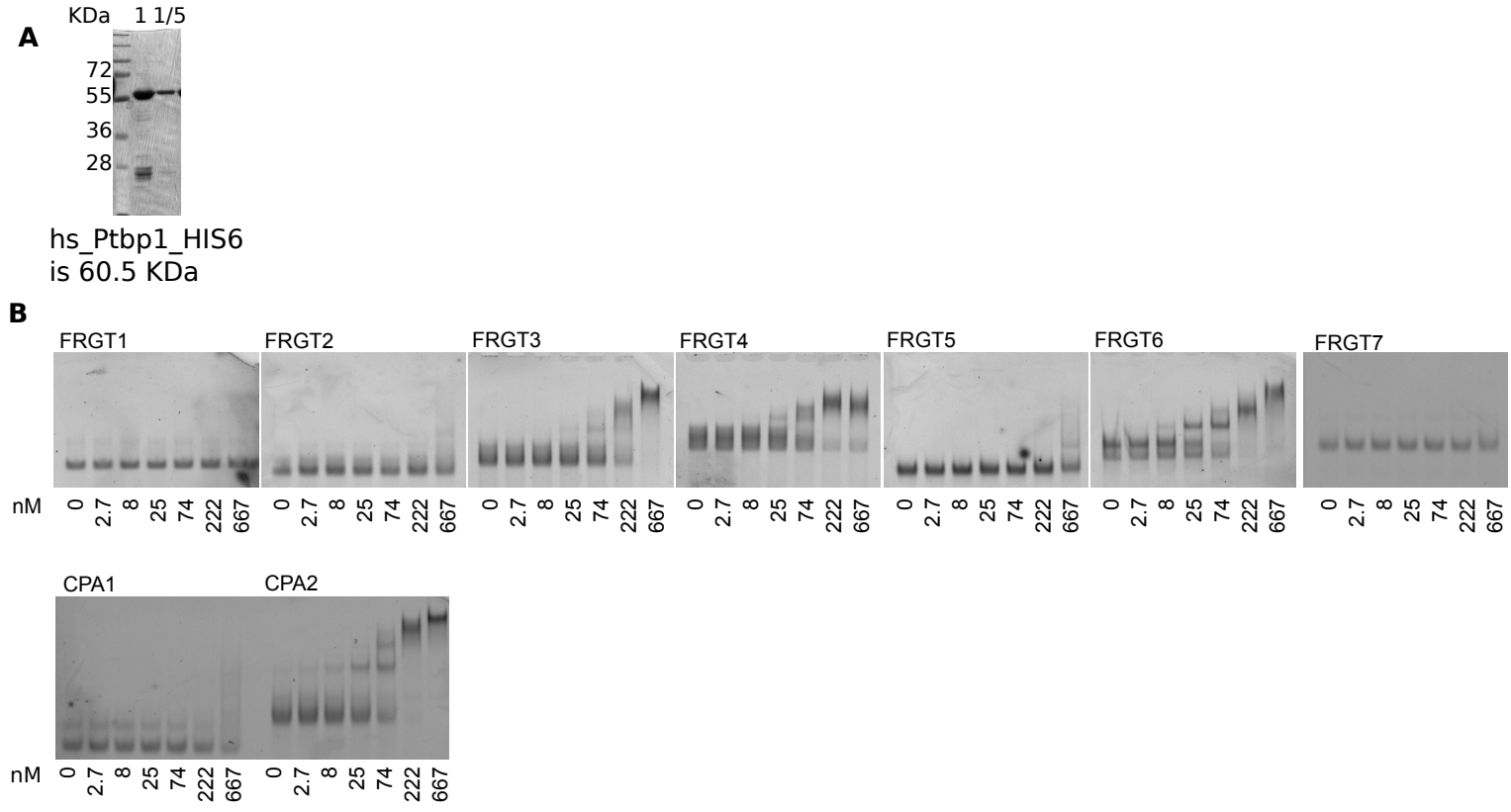
B TP63 Junction usage in TP63 expressing tissues



Supplementary Figure 2: Alternative junction usage for TP63 in GTEx data

A) Schematic of the TP63 gene model. The exons (boxes) and the quantified splice junctions (lines) are presented with their hg38 coordinates (green 5' splice sites, black 3' splice sites). Red ticks indicate cleavage and polyadenylation sites (CPA). The junctions pertaining to isoform γ are shown in red. B) junction usage among GTEx samples for the TP63 expressing tissues. The genomic coordinates of the junctions, the exons joined and the isoform involved (when specific) are shown on each graph.

Supplementary Figure 3



Supplementary Figure 3: Electrophoretic mobility shift assays

A) Validation of the purification of the recombinant human PTBP1 protein by gel electrophoresis and coomassie staining. B) Representative EMSA experiments conducted with fluorescently labeled RNA and recombinant PTBP1 proteins in increasing concentration (0 to 667 nM) (n >= 4). In each panel the lower bands in 0 nM PTBP1 correspond to free RNA probes.

1.2. “PTBP1 controls *TP63* alternative splicing in HNSCC tumour cells”: Discussion and perspectives

This work led to the first identification of a transacting factor, the RNA binding protein PTBP1, in the regulation of the decisive splicing event of *TP63*'s pre-mRNA that determines whether it will become a γ isoform, or any one of the $[\alpha, \beta, \delta]$ isoforms. The depletion of PTBP1 in human keratinocytes, in human HNSCC cells or in *Xenopus laevis* embryos invariably leads to the increase of the *TP63 γ* RNA. This dates this regulatory process to at least 350 million years ago, prior to the divergence of Lissamphibians. Interestingly, a recent publication highlighted the importance of pre-mRNA splicing regulation by SRSF3 in the evolutionarily conserved control of the biophysical properties of transcription factors. This therefore emphasizes that regulation of transcription factor activity may be efficiently mediated by alternative splicing regulation (Kawachi et al. 2021).

The conservation of the *TP63* gene structure and of its regulation is suggestive of their strong importance in development. Interestingly, the p63 γ specific peptide does not show strong conservation between species. This suggests that it is the fact that this isoform is devoid of C-terminal functional domains, rather than its novel peptide, that requires tight control. In adults, *TP63 γ* is only significantly expressed in skeletal muscles (Marshall et al. 2021). During embryogenesis, it is expressed during limb bud development (Kawata et al. 2017b), and is associated with the maturation of chondrocytes from proliferative cells to hypertrophic cells (Gu et al. 2013b; Yao et Wang 2013b). This isoform could therefore be specifically required at certain key points during development, in mesoderm derivatives rather than in ectoderm derivatives as *TP63 α* is. p63 γ can be integrated into heterotetramers with p63 α thanks to their oligomerisation domain, and shares the same DNA binding domain without any of the C-terminal domains. It is therefore likely that p63 γ can alter the effect of p63 α on its target sites in a dominant-negative-like manner, effectively serving as a damper to p63 α mediated effects.

At the most basic level, cancer can be described as a pathological loss of control of cellular homeostasis. This dysregulation classically involves gain of oncogenic functions and loss of tumour suppressor functions. These errors are often attributed to genetic abnormalities such as mutations, deletions or duplications. Once the oncogenic process has been initiated, activation and repression of numerous signalling pathways give cancer its trademark heterogeneity. A majority of proteins exist as several different isoforms dictated by alternative splicing. In some of these cases, shifting between two isoforms can

significantly impact cellular proliferation, migration, invasion or cell death. Shifting between isoforms to a more cancer-favourable proteome requires cancer cells to adapt the expression of regulatory factors. Alteration of alternative splicing programs during tumourigenesis can be illustrated by the recently described roles of multiple RNA binding proteins (SRSF3, RBM22, PTBP1 and RBM3) in the development of glioblastomas. The accumulation of these post-transcriptional regulators was associated with development of glioblastomas, modification of alternative splicing and defavorable cellular properties such as proliferation and cell migration (Fuentes-Fayos et al. 2020). Among the many different processes involved in tumour initiation and progression, the epithelial to mesenchymal transition is particularly prone to regulation by alternative splicing. Many effectors of the EMT can be expressed as either epithelial or mesenchymal isoforms. Shapiro and colleagues (Shapiro et al. 2011b), identified an EMT-associated alternative splicing signature that was dependent on a small number of RNA BP. Reversely, modification of transcription factor activity can alter expression of RBP. This may be illustrated more specifically by the association of individual RBPs with tumourigenesis. In this context, increased levels of c-myc promote an increase in polypyrimidine tract binding protein 1 (PTBP1) levels in gliomas. In turn, PTBP1 helps cancer cells proliferate by shifting the splicing of the pyruvate kinase PKM premRNA from PKM1 to PKM2 which switches the cellular energy production from oxidative phosphorylation to aerobic glycolysis and favour production of the metabolites necessary for transcription and replication (Cj et al. 2010). In breast, lung, colon and bladder tumours, the SR protein SRSF1 is upregulated and enhances proliferation and migration while repressing apoptosis. In oral squamous cell carcinomas showing advanced dysplasia or lymphatic metastasis, SRSF3 is upregulated and promotes the expression of EMT markers (Ghigna et al. 2005; Karni et al. 2007; Ezponda et al. 2010; Anczuków et al. 2012).

p63 α has been shown to repress the EMT in various types of cancer (Bergholz et Xiao 2012; Wu et al. 2014; Hu et al. 2017b; Lakshmanachetty et al. 2019a; Barbieri et al. 2006; Goto et al. 2014; Tucci et al. 2012; Tran et al. 2013a), and p63 γ has been shown to have a pro-EMT effect in breast cancer cells and in HPV E6/E7-expressing keratinocytes. Considering our identification of an association between the high proportion of *TP63* γ and poor patient survival in HNSCC, it seems quite plausible that PTBP1 could be used by cancer cells as a regulator of EMT. An increase in p63 γ could perhaps have a similar effect as the loss of p63 α which is frequently observed in advanced cases of HNSCC (Lakshmanachetty et al. 2019a).

More than half of human genes possess alternative promoters (Kimura et al. 2006). These act in combination with downstream alternative splicing events to generate multiple isoforms. In tissues, the occurrence of p63 γ seemed more often associated with TA (=P1) promoter usage. Global bioinformatic analysis of transcripts has shown that genes with multiple promoters tend to have more alternative splicing events, and the outcome of these splicing events is correlated to promoter use (Xin, Hu, et Kong 2008). At the functional level, the promoter's architecture influences splicing outcome (Cramer et al. 1997; 1999).

So what are the different possibilities that may explain such correlation? One can envisage that common factors are acting both on promoter usage and on alternative splicing to favour regulatory events that would be coupled. These events could be upstream events, signalling pathways for example, that are simultaneously targeting transcription factor and regulatory RNA binding proteins, therefore promoting a correlated regulation. These pathways could also act by promoting specific modifications of the chromatin or of transacting factors that would impact transcription and splicing. Alternatively modifications carried by transcription factors or RNAPol II could act on the recruitment of regulatory RNA binding proteins during transcription. Changes in the biophysical properties of the RNA polymerase itself, for example transcription speed or pausing, could impact alternative splicing. These modification of RNA polymerase characteristics could be general in a cell and still cause specific impact on different promoters or, they could be the consequence of alternative promoters usage favoring the loading of specific cofactors or by the distinct recruitment of factors involved in both transcription and splicing (KORNBLIHTT et al. 2004; Braunschweig et al. 2013; Merkhofer, Hu, et Johnson 2014). In prostate cancer for example, c-Myc binds to the promoter of Sam68, and by controlling transcription speed, favours the inclusion of an exon required for the functional full length mRNA (Caggiano et al. 2019). Alternative promoters are often used differentially between tissues or developmental time-points (Pankratova et al. 2005; Edupuganti et al. 2017; S.-H. Liang, Hassett, et Omiecinski 2005). In the case of *TP63*, the TA isoforms are mostly expressed in the germline cells, whereas the Δ N isoforms are expressed in the basal layers of many epithelia. In our work, we have shown that *TP63 γ* is hardly expressed in most tissues, but is robustly expressed in skeletal muscle cells. Furthermore, the majority of muscular *TP63* is of the *TATP63* isoform, suggesting that it is *TATP63 γ* which is expressed in this tissue, as reported recently by Marshall and colleagues in an independent analysis of GTEX dataset (Marshall et al. 2021). Through our experiments in human epithelial cell lines, we have shown that PTBP1 represses the

use of the *TP63 γ* -specific terminal exon. However, the induction of *TP63 γ* upon PTBP1 depletion in these cells remains modest. In these cells, *TP63* is almost exclusively expressed as Δ *NTP63* isoforms. This dichotomy of promoter use between skeletal muscle cells and the epithelial cells we have used could provide an additional explanation to the limited effect of PTBP1 depletion on *TP63 γ* levels, on top of the possible existence of *TP63 γ* splicing activators. It would be very interesting to compare PTBP1's impact on the maturation of *TATP63* pre-mRNAs to that which we have documented for Δ *NTP63* transcripts. This last approach could be undertaken in muscular cells where PTBP1 expression is low. One could use differentiable muscular cell lines or in vivo models of development such as *Xenopus* to address simultaneously the regulation and the functional consequences of isoform switching.

Chapter 2. Investigation of *TP63* isoform function in human epithelial cells

The different experiments presented as part of the first chapter highlighted the direct role of PTBP1 in the repression of *TP63*'s γ isoform. The analysis of patient RNA seq data from the HNSC TCGA database also revealed a correlative link between the amount of *TP63* γ present in a tumour sample and the duration of the patient's survival. This could mean that more *TP63* γ is present in tumours of worse prognosis as part of broader modifications to the RNA processing landscape, and could therefore potentially serve as a readout of these events. It could also mean that *TP63* γ somehow exerts a negative effect on survival, and could therefore warrant investigation as a causative event. The survival of HNSCC patients is directly related to whether they have developed metastases or not. The breaking point between a primary tumour and full on metastatic disease is thought to be dependant on the epithelial to mesenchymal transition (EMT) (Yun Zhang et Weinberg 2018). This is a process by which the adhesive apico-basal polarity of an epithelial cell is lost as it changes into a mesenchymal cell. EMT is promoted by many changes in the components and architecture of the cytoskeleton and associated adhesion molecules. Complex transcriptional (Cook et Vanderhyden 2020) and post-transcriptional programs (Shapiro et al. 2011b) are the driving forces in EMT. These changes provide affected cells with extra migratory capacities and, if coupled with the expression of matrix-remodelling enzymes, can also lead to increased invasive potential. The whole process of EMT is gradual and reversible, and there is therefore an EMT-MET continuum whereby cells can exist in any state from fully epithelial to fully mesenchymal (Pastushenko et al. 2018). In the case of metastasis, it is thought that a partial EMT allows some epithelial cells from the primary tumour to break loose and migrate to a distant site where they go through a MET phase to anchor themselves down and proliferate into a new tumour, acting as "cancer stem cells"(Saitoh 2018).

Associating p63 with EMT can at first seem surprising given its well described role in the differentiation and maintenance of epithelia. However, if we think in terms of isoforms, it begins to make more sense. p63 α has been described as an inhibitor of EMT, both in breast cancer and bladder cancer cells (Tran et al. 2013b; Yoh et al. 2016). In

agreement with this, HNSCC tumour progression is associated with a loss of *TP63* expression (Lakshmanachetty et al. 2019b). p63 γ has been described to promote the EMT process in both breast cancer cells (Lindsay et al. 2011b) and HPV transformed human keratinocytes (Srivastava et al. 2015; 2018b). p63 γ is the shortest of the p63 isoforms, and has the theoretical possibility to act as a dominant negative to p63 α . In physiological conditions, the α and γ isoforms show non overlapping expression in tissues: *TP63 α* is expressed in several epithelial tissues, whereas *TP63 γ* is expressed in muscles. We therefore hypothesized that the *TP63 γ* isoform could be involved in promoting certain mesenchymal properties in cells.

2.1. Comparison of EMT-related phenotypes between PTBP1 depleted and TGF- β treated HaCaT cells

Thanks to our identification of the repression of *TP63 γ* by PTBP1, we had the possibility to use siRNAs directed against PTBP1 as a means to modestly increase the abundance of *TP63 γ* in our cell lines. This had the advantage of simplicity since it did not require the labour intensive process of generating cell lines overexpressing *TP63 γ* , and would be a good model of physiologically possible levels of *TP63 γ* . We chose to proceed with HaCaT cells, as these have already been thoroughly used for the study of EMT (Räsänen et Vaheeri 2010; Hatta et al. 2018). They have the advantage of being sensitive to EMT induction by TGF- β , which we included as a positive control for EMT onset. The use of TGF- β to trigger EMT in HaCaT cells requires sensitization by serum-depletion. We therefore settled on comparing the effect of depleting PTBP1 with the effect of the addition of 2ng/mL of TGF- β on HaCaT cells cultured in 0.5% serum media. To appreciate the advent of an EMT, we combined western blotting with immunofluorescence imaging to look at modifications in abundance and/or localisation of commonly used markers. We chose to look at E-cadherin, Fibronectin, SRC and Slug as these markers have been previously associated with *TP63 γ* mediated EMT in keratinocytes (Srivastava et al. 2018b). After 96 hours of treatment, TGF- β treatment clearly resulted in Fibronectin and Slug accumulation as observed by Western-blot, while no effect was observed on either E-cadherin or SRC levels (**Figure 24.A**). As seen by immunofluorescence, TGF- β treated cells were depleted in E-cadherin staining at the membrane and Fibronectin was accumulating inside the cells (**Figure 24.B**, compare b and d). In siPTBP1 treated cells, while PTBP1 was clearly depleted as observed by western-blot (**Figure 24.A**, compare e and c), no modification of

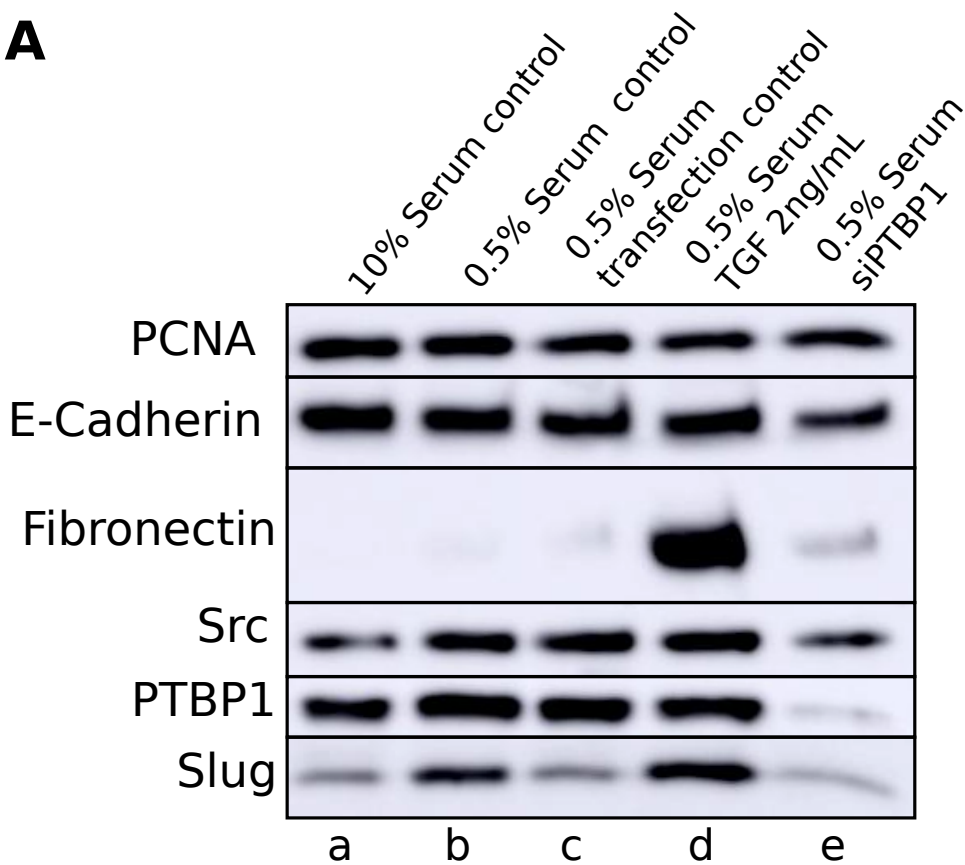
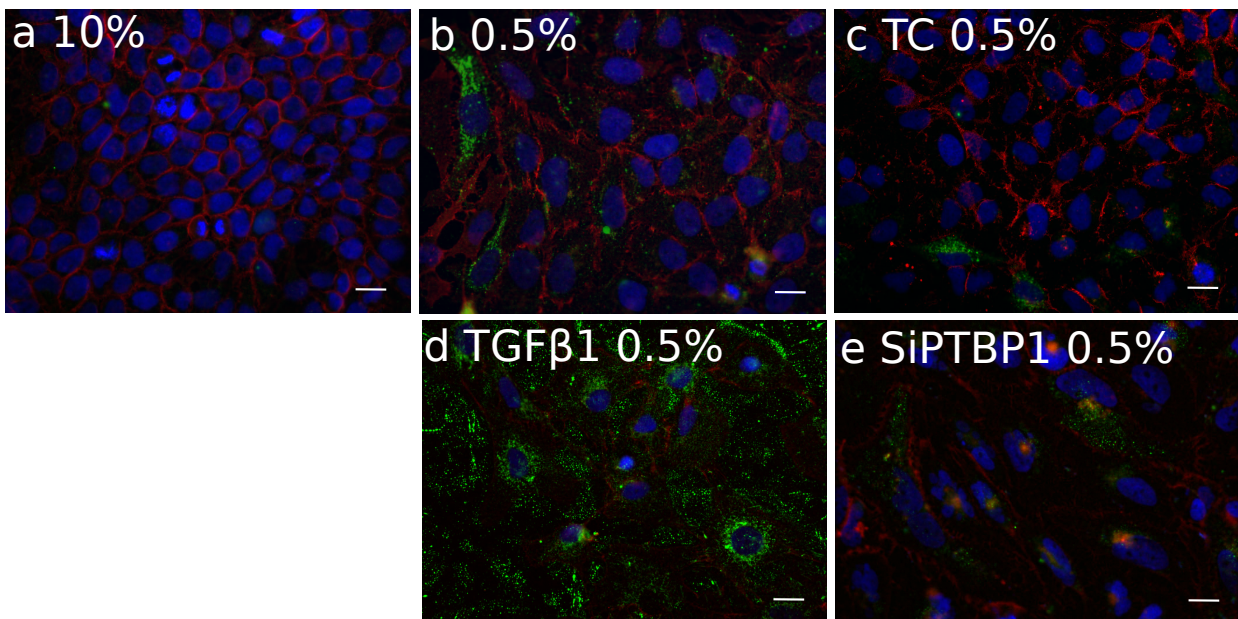
A**B**

Figure.24. Human keratinocytes (HaCat cells) were cultured in 10% or 0,5% serum containing medium and either treated with 2ng/mL TGF-β1 or transfected with PTBP1 siRNA. **A.** Total protein extracted from cells were analysed by western blot for E-cadherin, Fibronectin, Src, PTBP1, Slug and PCNA used as a loading control.(a) Control cells, 10% FCS. (b) Control cells, 0,5% FCS. (c) Transfection control cells, 0,5% serum. (d) TGF-β1 2ng/mL, 0,5% serum. (e) siPTBP1 transfected cells, 0,5% serum **B.** Immunofluorescence staining of fixed and permeabilised cells for E-cadherin (Red) and Fibronectin (Green) expression, counterstained with DAPI.

the EMT markers could be seen. The most obvious cellular phenotype following PTBP1 depletion is the drop in cellular density, with some cells becoming more elongated (**Figure 24.B**, compare c and e). Immunofluorescence analysis of E-cadherin showed it remains mostly present at the membrane (**Figure 24.A** compare b and c to e), and its levels seem slightly decreased as shown also by western blot analysis (**Figure 24.B** compare e to c). Interestingly E-cadherin positive spots appear around the nuclei following PTBP1 depletion (**Figure 24.A** compare c to e). This could correspond to its location in the endoplasmic reticulum(ER). Most of these spots are also Fibronectin positive, suggesting possible ER stress which we could hypothesize is a result of many aberrant splicing events following PTBP1 depletion. Fibronectin levels did not appear significantly different from serum depleted control cells (**Figure 24.A** Compare b and c to e). As a whole, while TGF- β could induce an EMT as confirmed by depletion of E-cadherin at the membrane and accumulation of Fibronectin, this experiment did not provide sufficient proof of an effect of PTBP1 depletion on the epithelial phenotype of the cells.

Similar PTBP1 depletion experiments were performed again on HaCaT cells in their normal serum conditions (10%). Both siPTBP1a and siPTBP1b could deplete PTBP1 from the cells (**Figure 25.B**), but once again Fibronectin levels were unaffected by the PTBP1 depletion as appreciated by western blot (**Figure 25.A**). E-cadherin levels did not change either as shown by western blot (**Figure 25.A**) and by quantification of immunofluorescence staining (**Figure 25.B, C**). These experiments therefore provide no evidence tying PTBP1 depletion to the onset of EMT in HaCaT cells.

The targets we chose were however markers that are known to be affected when cells transition strongly towards the mesenchymal phenotype (Scanlon et al. 2013). It is possible that we would have missed a more subtle, partial-EMT triggered by PTBP1 depletion by limiting ourselves to these. The fact that we did not reproduce the EMT phenotype described following *TP63 γ* overexpression in (Srivastava et al. 2018b) is likely down to the vast difference in *TP63 γ* levels between our respective experiments. Indeed, by overexpressing *TP63 γ* using a pENTR11 based vector, the authors achieved p63 γ levels which were dramatically stronger than the endogenous p63 α . Following PTBP1 depletion, *TP63 γ* levels in our cells still make up less than 10% of the overall expressed *TP63* isoforms, as illustrated in the first chapter.

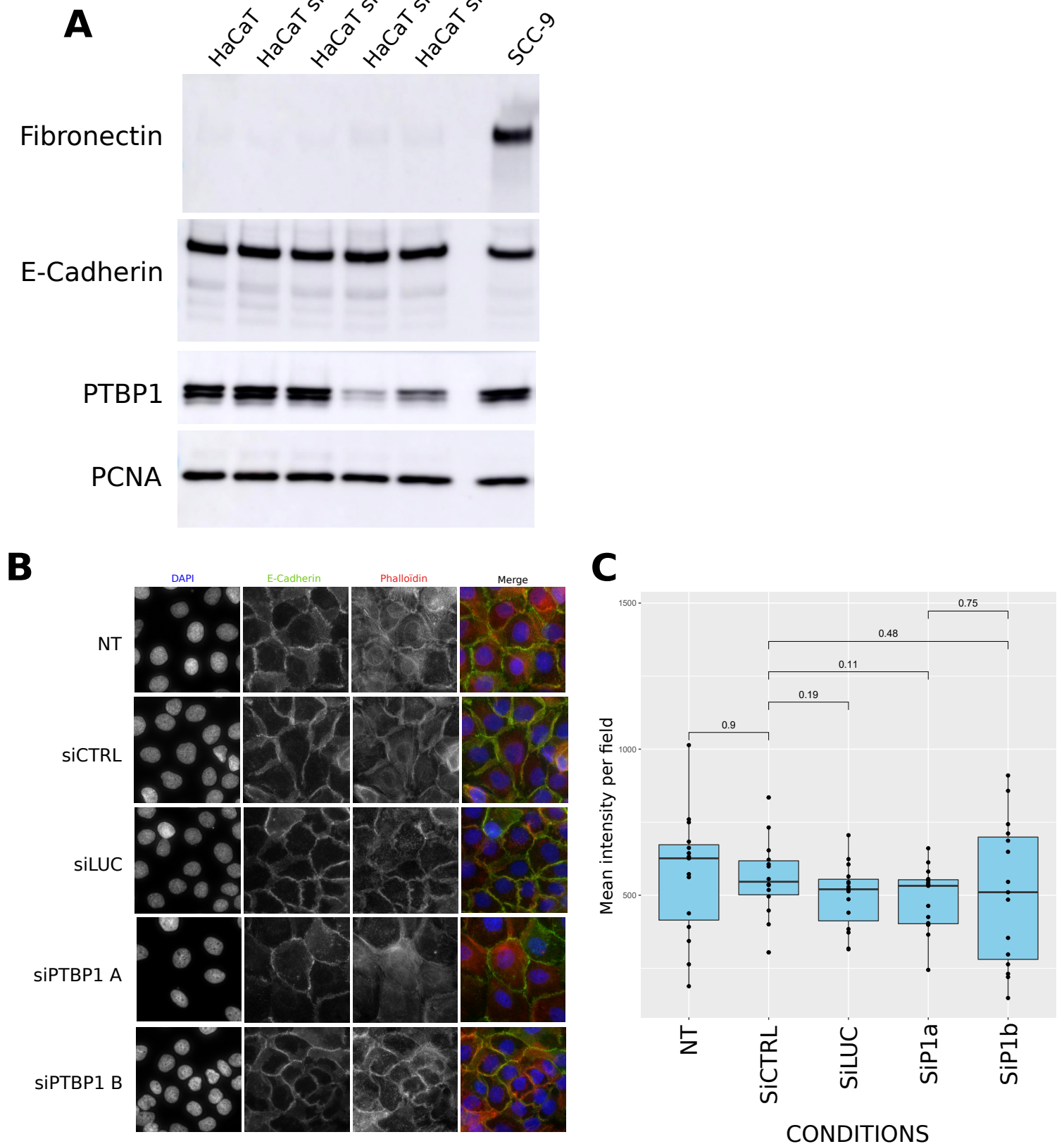


Figure.25. Human keratinocytes (HaCat cells) were cultured in 10% serum media and transfected with either PTBP1 siRNAs a or b or with control siRNAs. Cells were harvested after 96 hours of treatment. SCC9 cell extracts are loaded as control. **A.** Total protein was analysed by western blot for E-cadherin, Fibronectin and PTBP1, with PCNA used as a loading control. **B.** Immunofluorescence images were taken following E-cadherin, phalloidin and DAPI staining. Images are the result of stacked Z-slices. **C.** Immunofluorescence staining of E-cadherin was performed, followed by measurement of E-cadherin staining intensity at the cell membrane using imagej software. For each tested condition, 15 fields containing an average of 10 cells were imaged and analysed. Statistical significance of the difference of means was calculated by student's T test.

2.2. Comparison of EMT-related phenotypes between PTBP1 depleted and TGF- β treated SCC-9 cells

SCC-9 cells (Tongue squamous cell carcinoma) offer the advantage of being naturally susceptible to engage into partial EMT, and do not require serum starvation to respond to TGF- β treatment (Puram et al. 2017b). One of the limiting factors in identifying an effect of the *TP63 γ* isoform is its low expression, even following PTBP1 depletion. PTBP1's ortholog PTBP2 is known to be induced following PTBP1 depletion (Boutz, Stoilov, et al. 2007; Makeyev et al. 2007; Rachel Spellman, Llorian, et Smith 2007) and shares a partial overlap of its functions with PTBP1 (Vuong et al. 2016). Therefore phenotypes associated with depletion of PTBP1 could be partially masked by the redundant functions of PTBP2. We therefore sought to co-deplete PTBP1 and PTBP2 to favour accumulation of *TP63 γ* . For this, SCC-9 cells were transfected with siRNAs against *PTBP1* (*SIP1 is SiPTBP1a*), *PTBP2* (*SIP2*), *TP63 γ* (*SIG*) or combinations of each (**Figure 26**). While siP1 depleted specifically PTBP1 and concomitantly increased PTBP2 levels compared to non-transfected cells, siP2 could deplete both PTBP2 and most remarkably PTBP1. Depletions of *PTBP1* and *PTBP2* was confirmed by RT-qPCR when compared to non-transfected SCC-9 cells. Co depletion experiments with siP1 and siP2 efficiently depleted PTBP1 and reduced levels of PTBP2 to the levels observed in control cells. *TP63 γ* levels seemed marginally increased when both siRNAs against *PTBP1* and *PTBP2* were combined and were specifically reduced upon treatment with *SIG* alone or in combination with *siP1P2*. Therefore, we could rule out a strong effect of PTBP2 re-expression following PTBP1 depletion on the overall levels of *TP63 γ* . SiG led to some downregulation of overall *TP63* expression and of *TP63 α* expression. It would probably be worth investigating whether the effect of *TP63 γ* depletion on *TP63* is the result of an indirect effect of the siRNA on the stability of *TP63* pre-mRNA or if down-regulation of p63 γ protein itself is responsible for this effect. This would set *TP63 γ* as a factor promoting *TP63* alpha expression.

In our continued search for potential effects of PTBP1 knockdown on EMT and similar to what we did on HaCaT cells, we treated SCC-9 cells with 2ng/mL TGF- β for 48, 96 or 144h. We then compared the impact of the addition of TGF- β with the impact of siRNA mediated depletions of PTBP1 and PTBP2 on the expression of various EMT and partial EMT genes using RT-qPCR (**Figure 27**). Partial-EMT (P-EMT) genes are expressed by cells which have started to differentiate from an epithelial state, undergoing the transition towards the mesenchymal state without necessarily fully committing to it

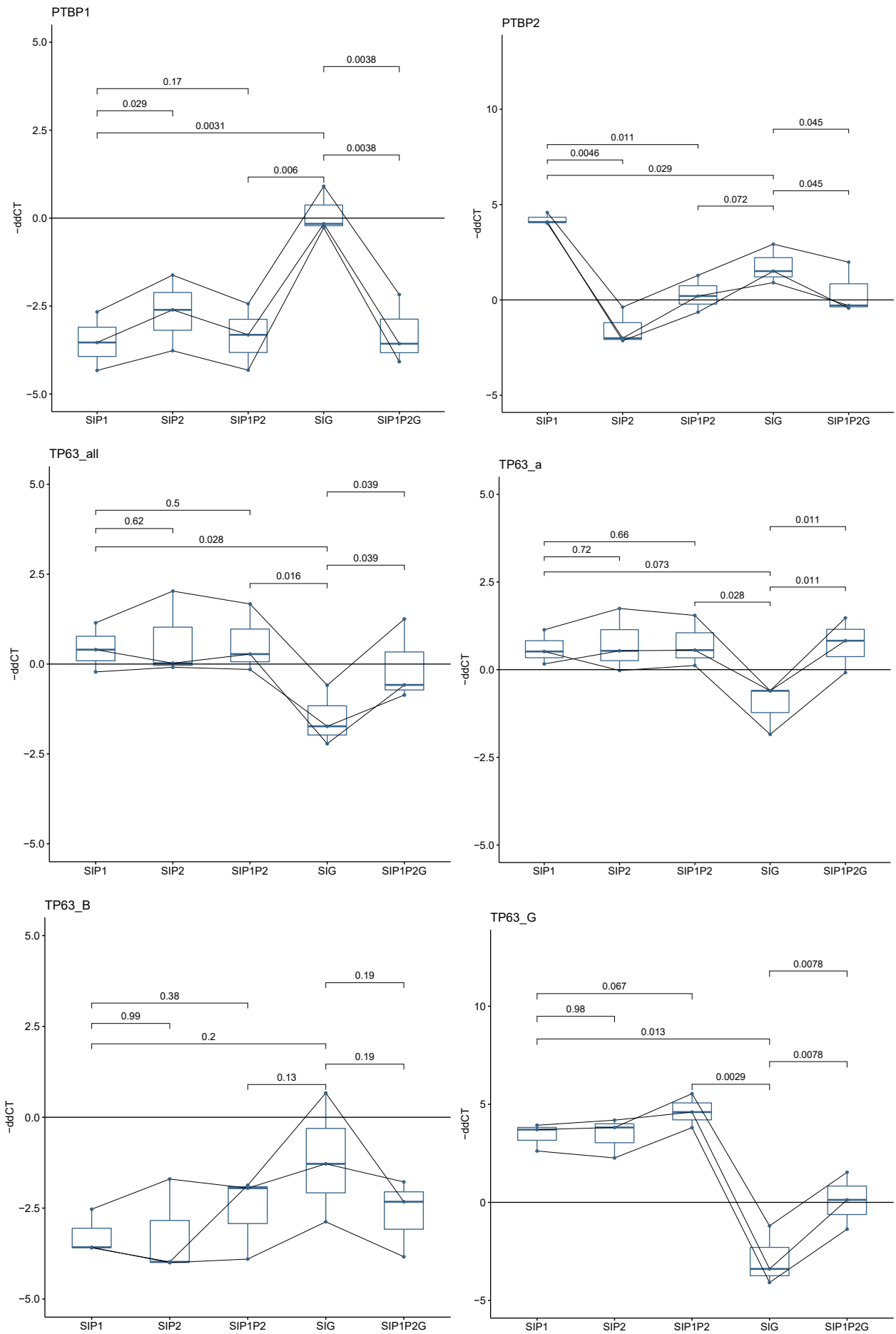


Figure.26. SCC-9 cells were transfected with siRNA against PTBP1 (SiP1), PTBP2 (SiP2), TP63 γ (SiG) or combinations of each. Gene expression of PTBP1, PTBP2, and TP63 isoforms was measured by RT-qPCR and plotted as -ddCT, using non transfected cells as a control.

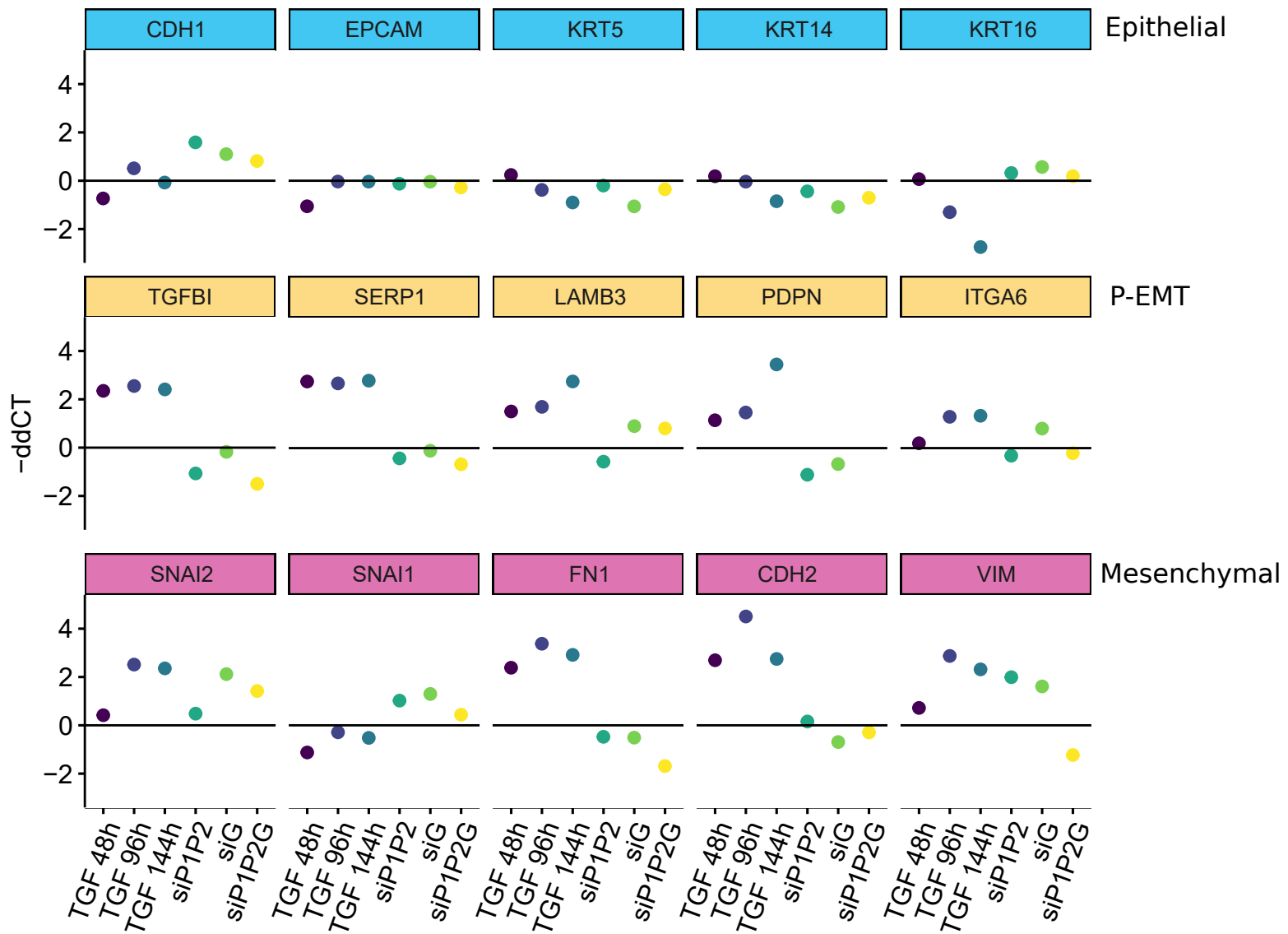


Figure.27. SCC-9 cells were cultured in 10% serum containing medium and either treated with 2ng/mL TGF- β 1 or transfected with siPTBP1 and siPTBP2, siTP63 γ or a combination of the three. Gene expression was measured by RT-qPCR and analysed by the ddCT method. The reference gene is RPLP0. siRNA depleted samples were normalised to untransfected cells, and each TGF- β treated sample was normalised according to its associated untreated control.

(Figure 23). The epithelial phenotype of cells is characterised by an apico-basal polarity, and low mobility through tight cell-cell and cell-basement membrane junctions **(Figure 23)**. The mesenchymal phenotype is in complete opposition: cells are motile and invasive, they have a front-back polarity (Etienne-Manneville 2008), and do not adhere strongly to cells or the matrix. P-EMT cells can share any of these properties, and in HNSCC, cells in between the two states tend to retain epithelial markers including E-cadherin as they acquire certain mesenchymal markers (Pal et al. 2021b). We chose to look at three groups of markers: *CDH1* (E-cadherin), *EPCAM*, *KRT5*, *KRT14*, *KRT16* were used as markers of the epithelial state; *TGFBI*, *SERP1*, *LAMB3*, *PDPN* and *ITGA6* were used as P-EMT markers; and *SNAI2*, *SNAI1*, *FN1*, *CDH2* and *VIM* were used as mesenchymal markers (Puram et al. 2017b).

We first established the differential expression of the three sets of markers upon TGF- β treatment as a way to induce EMT. Amongst the epithelial markers, only *KRT16* was decreased in the days following TGF- β treatment. This is in corroboration with work indicating that *KRT16* levels are decreased in a subgroup of HNSCC patients with poor metastasis-free survival (Jung et al. 2013). Among P-EMT markers, *TGFBI* and *SERP1* were increased to their maximum observed level as soon as 48h after TGF- β treatment, *LAMB3* and *PDPN* showed a more gradual increase, with their highest levels reached at 144h after TGF- β treatment. These genes are therefore probably part of a second differentiation step in the context of a TGF- β -induced EMT and are likely expressed in response to the activation of earlier targets. Despite being associated with increased migration and invasion in HNSCC (Kinoshita et al. 2013), as well as hepatocellular carcinomas and metastatic breast cancer (Lv et al. 2013; Brooks et al. 2016) *ITGA6* levels showed no changes in any of the tested conditions. The mesenchymal markers *FN1* (*Fibronectin*) and *CDH2* (*N-Cadherin*) were surprisingly expressed as soon as 48h after TGF- β treatment as evidenced in **Figure 27**, which highlights the fact that the transition from an epithelial to a mesenchymal phenotype does not necessarily require the cells to progressively move through intermediary states. Considering SCC-9 cells are naturally heterogeneous, with a subset expressing P-EMT genes in normal growth conditions (Puram et al. 2017b), it is also possible that it is these cells that were rapidly pushed by the TGF- β treatment towards a more mesenchymal state revealed by *FN1* and *CDH2* increased expression. *SNAI2* and *VIM* showed a more progressive induction, and *SNAI1* was not affected at all. Therefore TGF- β treatment induces an EMT in SCC9 cells with rapid induction of P-EMT markers and some EMT markers.

Depleting PTBP1 and PTBP2 had no impact on expression of the epithelial markers, and only a moderate impact on the EMT or P-EMT markers tested. Only an increase in *VIM* expression could be observed. Depleting *TP63y* had no effect on epithelial markers, no effect on P-EMT markers and led to some induction of *SNAI2* and *VIM* expression suggesting, counter-intuitively, that *TP63y* could be pro-epithelial. Altogether, downregulation of *PTBP1* and/or *TP63y* showed no particular pattern in EMT marker expression.

In colorectal cancer cells, PTBP1 stimulates EMT and migration by repressing the expression of *ATG10* (Jo et al. 2017). In lung carcinoma cells, PTBP1 provokes the skipping of exon11a in Mena pre-mRNA to produce a Mena isoform that promotes migration and invasion. PTBP1 levels are significantly increased in lung adenocarcinoma compared to normal tissue, and contributes to its metastatic ability (S. Li et al. 2019). Adenocarcinoma cells typically do not express $\Delta NTP63$ (Bishop et al. 2012b). Considering these results, it is likely than any pro-EMT effects resulting from an increase of p63y would be lost within the many consequences of PTBP1 depletion, some of which could be opposed to the EMT onset. From these experiments, we can therefore conclude that depleting PTBP1 and 2 does not provoke an epithelial to mesenchymal transition, but we cannot say whether an increased expression of *TP63y* outside of this context would impact the EMT.

2.2. Identifying p63 targets in HNSCC cells

Considering we have so far not been able to identify a role of p63's γ isoform in the EMT process, we wished to identify some of its target genes in a broader context and test for any overlap with known P63 α target genes.

Our first approach was to use PTBP1 and PTBP2 depletion to induce *TP63y* expression and combine it with with *TP63y* depletion to try to rescue the changes in expression that would therefore identify differentially expressed genes in SCC-9 and FaDu cell lines (**Figure 28**), as we had done previously for the study of the EMT. For this, we chose a short list of genes reputed to be targetted by p63 in the literature: *CDKN1A* (LeBoeuf et al. 2010), *FGFR2* (Research 2013), *MDM2* (Ghioni et al. 2002), *PERP* (Ihrie et al. 2005), *RUNX1* (Masse et al. 2012) and *SOX4* (Qu et al. 2018). We first controlled PTBP1 depletion in FaDu and SCC9 cells by western blot. Single PTBP1 or dual PTBP1/PTBP2 depletion led to a reduction in PTBP1 levels in all of the conditions (**Figure**

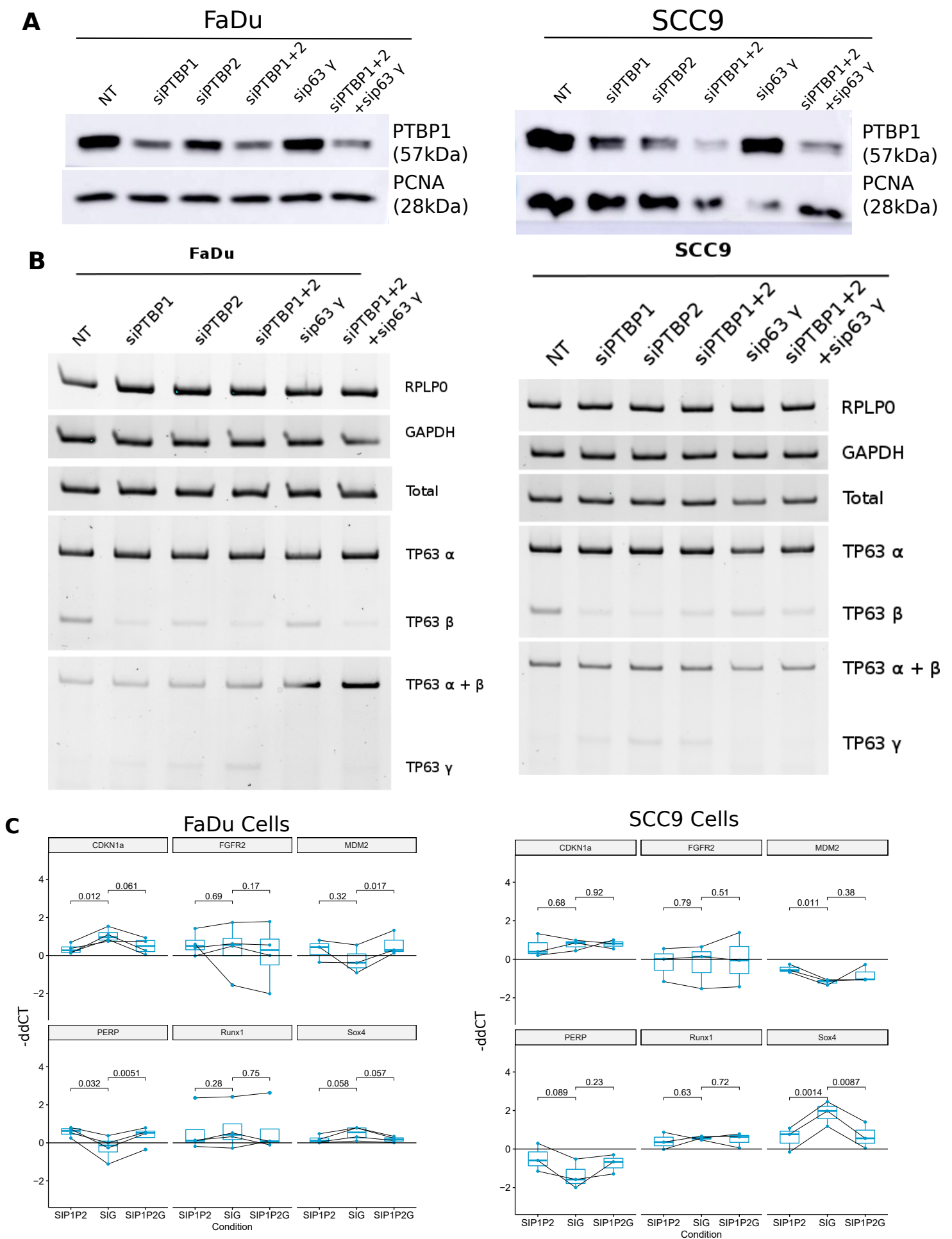


Figure.28. FaDu or SCC-9 cells were transfected with siPTBP1 and siPTBP2, siTP63 γ or a combination of the three. Protein levels were assessed by western blot for the indicated proteins (A), TP63 isoforms expression was analysed by RT-PCR and gene expression was measured by RT-qPCR for the indicated target and plotted as the ddCT method. Reference gene is RPLP0, reference sample is untransfected cells.

28.A). Upon depletion of PTBP1, PTBP2 or PTBP1+ PTBP2, an increase in *TP63 γ* was observed in FaDu and SCC9 cells as visualized by RT-PCR (**Figure 28.B**). This increase was abolished when cells were treated with siRNA against *TP63 γ* . In FaDu cells, depletion of *TP63 γ* led to a significant increase in *CDKN1A* and a significant decrease in *PERP* when compared to siP1P2, the most extreme conditions with respectively the least and the most *TP63 γ* (**Figure 28.C**). In SCC9 cells, only *SOX4* was significantly increased upon depletion of *TP63 γ* when compared to siP1P2. Co-depletion of *PTBP1/2* and *TP63 γ* restored *SOX4* levels to the levels in PTBP1/2 depleted cells. *P63 γ* could therefore be a negative regulator of *SOX4* in SCC-9 cells, and of *CDKN1A* in FaDu cells. This corresponds with the inhibitory role of *P63 α* on these two targets as stated in the literature, and would suggest that *p63 γ* does not have an antagonistic function in targetting these genes.

We tried to alter *TP63 γ* levels by modifying availability of the regulatory RNABPs PTBP1 and PTBP2. One of the potential caveats of this approach is to hinder their potential impact on *TP63* by altering other PTBP targets. Therefore, to more directly assess the potential function of *P63 α* and *P63 γ* we designed siRNAs targeting each of these isoforms specifically. Using these siRNAs, we sought to determine whether *p63* target genes were influenced by *p63 α* , *p63 γ* , or perhaps both. For this, we chose to use SCC-9 cells which express low levels of *p63* and A253 cells which express high levels of *p63* (see chapter 1). Efficient knockdown of *p63* was controlled at the protein (**Figure 29.A**) and RNA levels (**Figure 29.B**). As a readout of *p63* transcriptional activity we measured the RNA levels of *CDKN1A*, *MDM2*, *PERP* and *SOX4* (**Figure 29.B**), which had shown somewhat promising results previously. As *p63 α* has been linked with an anti-EMT role, we also looked at Fibronectin, E-Cadherin and Vimentin levels by western blot (**Figure 29.A**). SCC-9 cells typically express more mesenchymal markers as illustrated here by their higher expression of Fibronectin than A253 cells in the same conditions. Depleting *TP63 α* or *γ* had no impact on the expression of these EMT markers, suggesting that though *p63 α* may protect against EMT, inhibiting its expression is not sufficient to induce one. As was previously observed in **Figure 26**, transfection of any of the siRNA targeting *TP63 γ* led to partial but efficient depletion of *p63 α* as can be seen in (**Figure 29.A**).

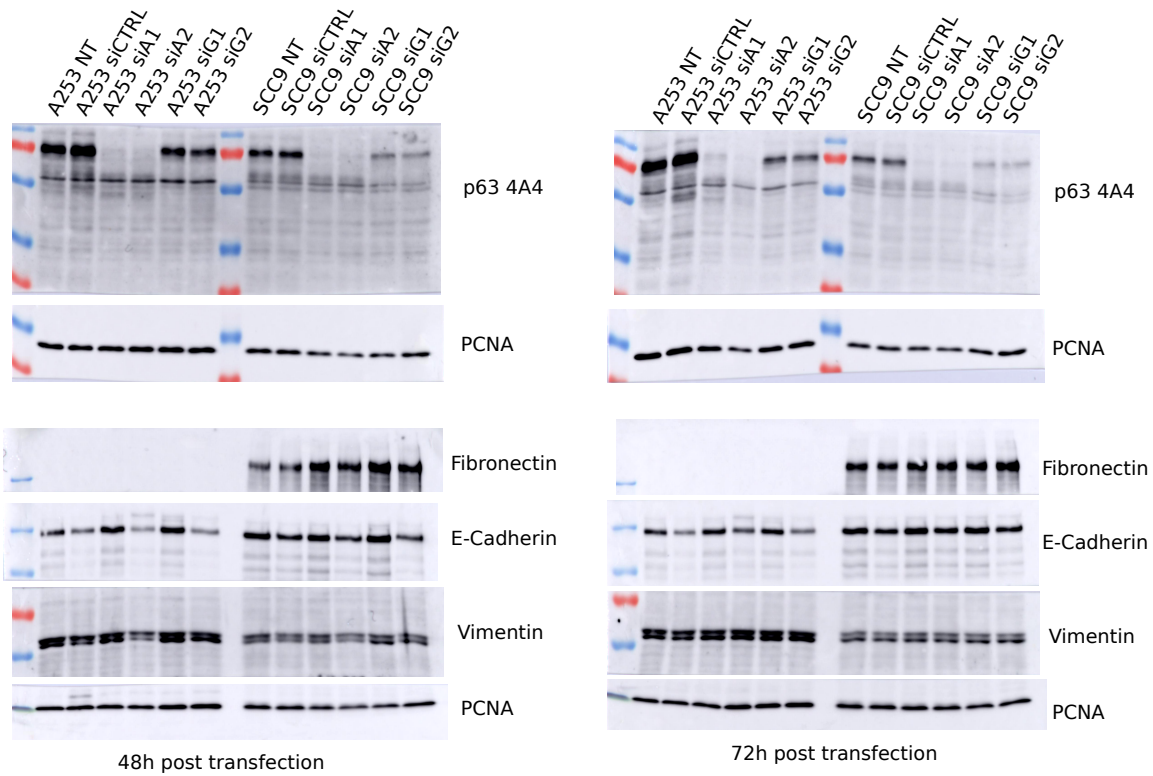
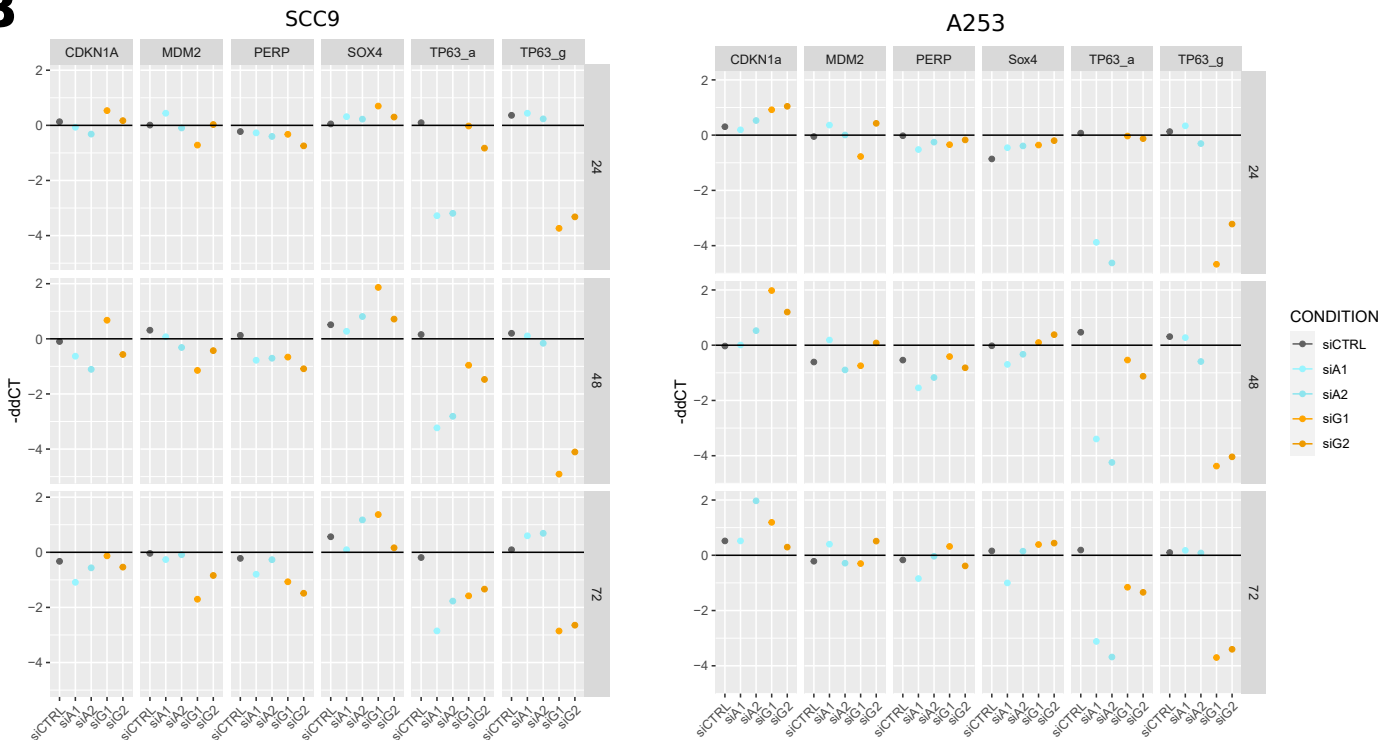
A**B**

Figure.29. SCC9 and A253 cells were transfected with the indicated SiRNA targeting either the alpha or the gamma TP63 isoforms. **A.** Total protein was analysed by western blot at two different time points after transfection to control for P63 and mesenchymal markers expression as indicated on the side of the blots. **B.** Expression of potential TP63 target genes was assessed by RT-qPCR and compared to untransfected cells. RPLP0 serves as reference gene.

When measuring expression of potential p63 target genes, in SCC-9 cells, we had previously shown an increase of SOX4 expression following *TP63 γ* knockdown using siRNA siG1 (**Figure 28**). Here, only siG1 showed this effect. Considering both *TP63 γ* siRNAs efficiently deplete its RNA levels, it's likely that the increase in SOX4 was merely an off-target effect of siTP63 γ n°1. There is no effect of *TP63* depletion on SOX4 in the A253 cells. In SCC9 cells, *TP63 γ* knockdown but not *TP63 α* knockdown leads to a progressive decrease of MDM2 levels from 24 to 72h after treatment. Again, this was not observed in A253 cells. The increase in CDKN1A observed previously in FaDu cells following *TP63 γ* depletion seems conserved in A253 cells until 48h, but not at 72h, and not in SCC-9 cells. Overall, the limited fluctuation in expression levels of the tested targets coupled with the single experiment make it impossible to robustly conclude to an effect on any of these targets, and taking the matter further would require repeating the experiment.

The most striking conclusion of these experiments is actually not on p63's targets, but on p63 itself. Upon depleting *TP63 γ* RNA, *TP63 α* RNA is also depleted (**Figure 29.B**), as well as the p63 α protein (**Figure 29.A**). This effect is reproduced in both A253 and SCC-9 cells, and with two different siRNAs. Several mechanisms could potentially explain this effect.

p63 activates its own transcription through binding to C38 and C40 enhancer elements as shown in mouse primary keratinocytes (Antonini et al. 2015). p63 self-activation is also mediated by its binding to its own promoter. Both Δ Np63 α and γ , as well as their TA counterparts are capable of activating a Δ NTP63 minimal promoter in Ptk2 cells (Potorous tridactylus kidney epithelial cells) (Romano, Birkaya, et Sinha 2006). TAP63 γ also stimulates Δ NTP63 expression in non-small cell lung carcinoma cells and in immortalised mammary epithelial cells (N. Li et al. 2006). A potential argument against this theory is the fact that in SCC-9 cells overexpressing *TP63 γ* , *TP63 α* levels are not increased at the RNA or protein levels (**Figure 30. A and B**). However, expressing ectopically Δ NP63 γ in HeLa cells activates the transcription of the endogenous Δ NTP63 in a DBD-dependant manner (Antonini et al. 2006). In HeLa cells *TP63* is not expressed under normal circumstances, but it is in SCC-9 cells (**Figure 30.C**). It therefore seems logical that adding p63 γ to the *TP63* locus would have more effect on a naive locus than adding it to an already transcriptionally active one.

RNA interference by siRNA is usually used as a tool for post-transcriptional repression of specific transcripts, by triggering their degradation. During transcription, siRNAs also have the possibility to influence gene expression by directing histone H3K9,

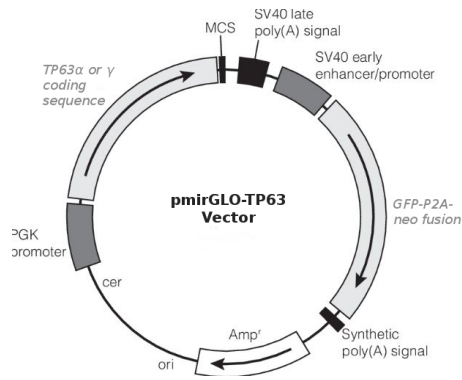
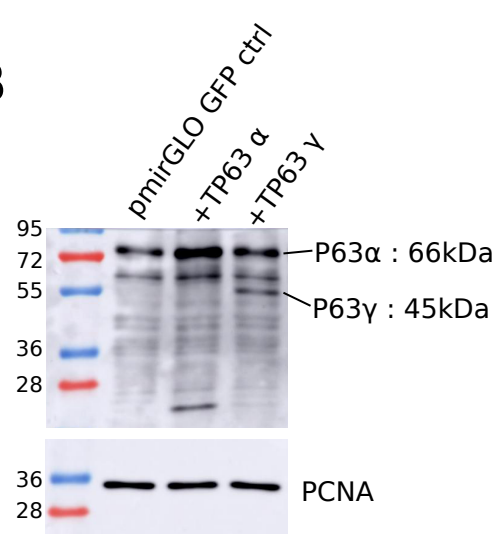
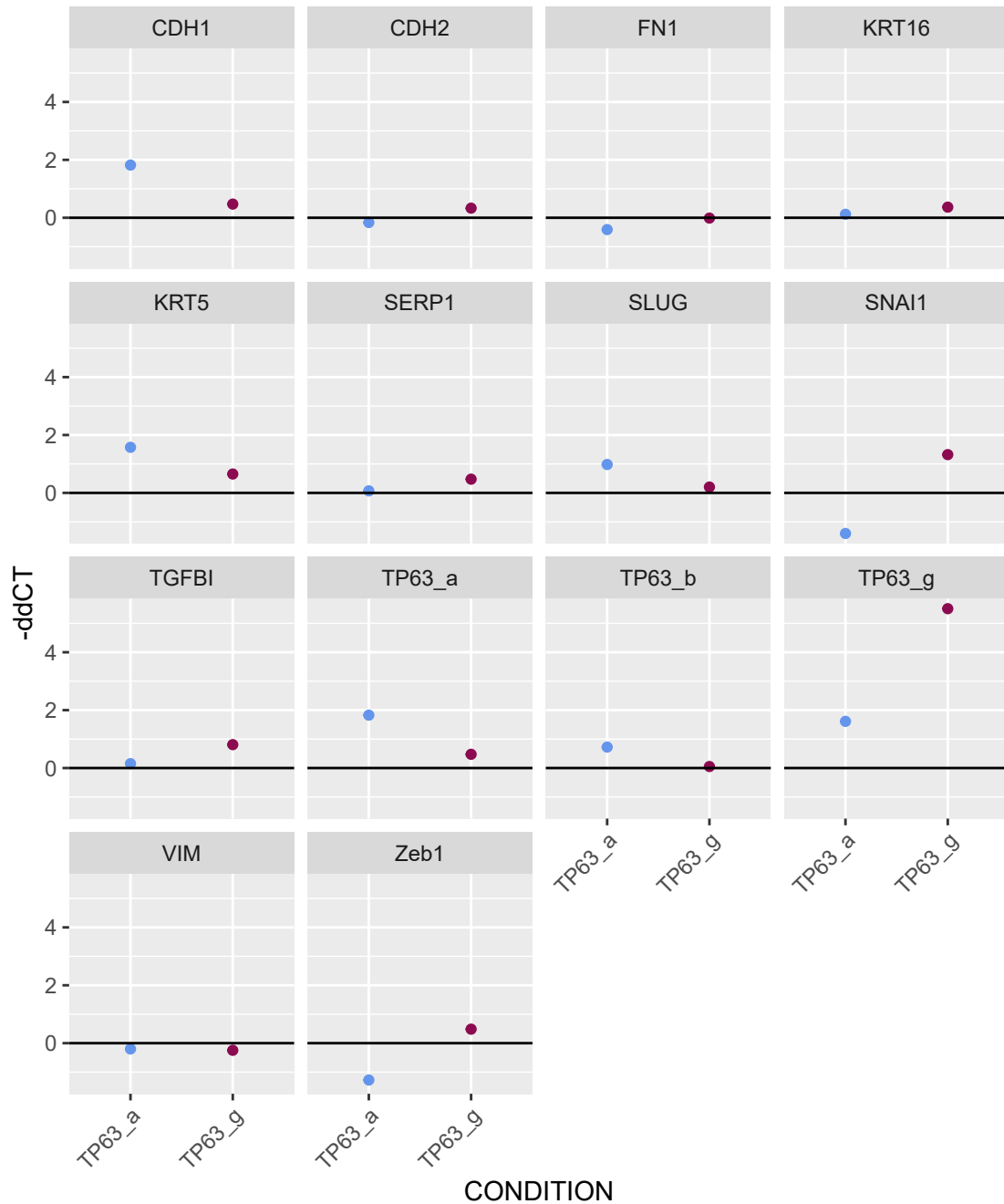
A**B****C**

Figure.30. SCC9 cells were transfected with pmirGLO GFP vectors containing the TP63 α or TP63 γ coding sequences. Stable multiclonal cell lines were subsequently derived and used for experimental purposes. **A.** Schematic representation of pmirGLO derived TP63 α or TP63 γ expression vectors. **B.** Total protein was analysed by western blot to control P63 isoforms expression in the newly established SCC-9 cell lines. **C.** Expression of epithelial or mesenchymal related markers was assessed in SCC9 cell lines by RT-qPCR and compared to the empty vector cell line.

H3K27, and DNA methylation. This effect seems restricted to siRNAs targeting promoter regions (Hawkins et al. 2009). The interaction between Ago2 and the RNA pol II suggests that siRNAs targeting sequences downstream of the promoter could also have an impact on the dynamics of their transcription. These mechanisms have been reviewed by (Weinberg et Morris 2016; Castel et Martienssen 2013). If *TP63y* siRNA made its way into the nucleus, there is therefore a possibility that it could impact the transcription of the *TP63* gene. This hypothesis would have deep consequences on the regular use of siRNA for isoform specific depletion, not only of *TP63*, but also in general.

Since *TP63 α* is downregulated at both the RNA and protein levels, we can rule out any stabilizing action of γ on α at the protein levels without transcriptional consequences. It does remain possible that p63 γ stabilizes p63 α proteins, which in turn activate *TP63* transcription. Removing p63 γ in this context would therefore lead to a drop in p63 α proteins, followed by a drop in overall TP63 RNA, which fits with our observations.

2.3. Using *TP63 α* and *TP63y* overexpressing SCC-9 cells to identify differential targets and functions

Whatever the mechanism by which $\Delta NTP63\alpha$ is downregulated following *TP63y* siRNA, the conclusion is the same: it is impossible to confidently identify isoform-specific targets of *TP63* with this knock-down method. We therefore set out to test a gain of function approach by creating cell lines stably overexpressing $\Delta NTP63\alpha$ or $\Delta NTP63y$. We initially chose to work with A253 and SCC-9 cells, as their initial levels of *TP63 α* are respectively high and low, and SCC-9 cells already express some mesenchymal markers whereas A253 cells do not. However, we could not establish cell lines from A253 cells, and were forced to limit our study to SCC-9 cells. $\Delta NTP63$ isoform expression was driven by a PGK promoter in order to limit the overexpression to near-physiological levels (**Figure 30.A**). **Figure 30.B** presents a western blot analysis of p63 expression in the 3 situations, empty vector, $\Delta NTP63\alpha$, or $\Delta NTP63y$. As shown using the 4A4 antibody, $\Delta Np63\alpha$ is already expressed in empty vector cells. The levels of $\Delta Np63\alpha$ increases in *pmirGLO* $\Delta NTP63\alpha$ cells. An immunoreactive protein of low molecular weight is also detected in these cells. Considering the location of the 4A4 epitope in the N-terminal region of $\Delta Np63$ (Nekulova et al. 2013a), this band of about 20kDa is most probably the result of a cleavage occurring in the DNA binding domain of $\Delta Np63$. *pmirGLO* $\Delta NTP63y$ specifically drives the accumulation of a protein with a size and an immunoreactivity coherent with

Δ Np63 γ . **(Figure 30.B)**. RT-qPCR quantification of Δ NTP63 α , or Δ NTP63 γ expression indicate that TP63 γ was increased 32 fold, and TP63 α , which is already reasonably well expressed, by 4 fold **(Figure 30.C)**. Western blot analysis is also coherent with this estimate **(Figure 30.C)**. Due to time constraints, we chose to evaluate proliferation and migration capacities with multi-clonal cell lines rather than isolate individual clones. First of all, the generated cell lines did not display any strong morphological changes compared to wild type cells **(Figure 31.A)**. Proliferation of the cells was analysed by nucleus counting for 96 hours. Naive, control, Δ NTP63 α , or Δ NTP63 γ cells all have a similar proliferation rate (n=4, **Figure 31B**). Migration properties of the cells were evaluated using Ibidi cell culture inserts. Cells seeded at equivalent density were videorecorded after wounding and the percentage of wound closure plotted against time **(Figure 31.C)**. SCC-9 control and naive cells were the slower migrating cells. Both Δ NTP63 α , or Δ NTP63 γ were faster to close the wound. However these experiments suffer from a quite high variability more specifically on the Δ NTP63 α overexpressing cells that preclude drawing any conclusion. Albeit non significant, the tendency was towards a higher migration rate for TP63 overexpressing cells, a conclusion that would be rather counter intuitive **(Figure 31.D)**. It is possible that clonal analysis of the cells would allow for a more statistically robust analysis to be conducted. Finally we assessed using RT-qPCR some markers or known targets of p63. **(Figure 31.E)**. Having at hand cells that overexpress Δ NTP63 α , or Δ NTP63 γ , we chose to compare those overexpressing cells with SCC-9 cells following depletion of TP63 isoforms. An increase in p63 α strengthens the epithelial identity of SCC-9 cells, as shown by the increase in CDH1 and KRT5, while having no effect on mesenchymal markers (FN1, SNAI2, SNAI1, VIM, ZEB1) or P-EMT markers (SERP1, TGFB1). Overexpressing TP63 γ had no effect on any of these markers **(Figure 30.C)**, and is therefore not sufficient to induce an EMT in these cells.

Since we had limited success in identifying robust changes in the expression of the p63 targets that we had previously selected, we sought to test some others **(Figure 31.E)**. *HERC3* is directly transactivated by Δ Np63 α in MCF10A and FaDu cells (Y. Chen et al. 2018). *SERPINB13* is downregulated in HNSCC and is associated with a poor clinical outcome (de Koning et al. 2009). *LAD1* is increased in HNSCC and promotes cellular growth and invasion (Abe et al. 2019). Both genes have been identified as p63 targets (Riege et al., s. d.). *HERC3* and *LAD1* levels were unaffected by both siRNA knockdowns and overexpression of TP63 α and γ **(Figure 31.E)**. *SERPINB13* expression seemed to be slightly decreased by TP63 α knockdown and increased by TP63 α overexpression. This is

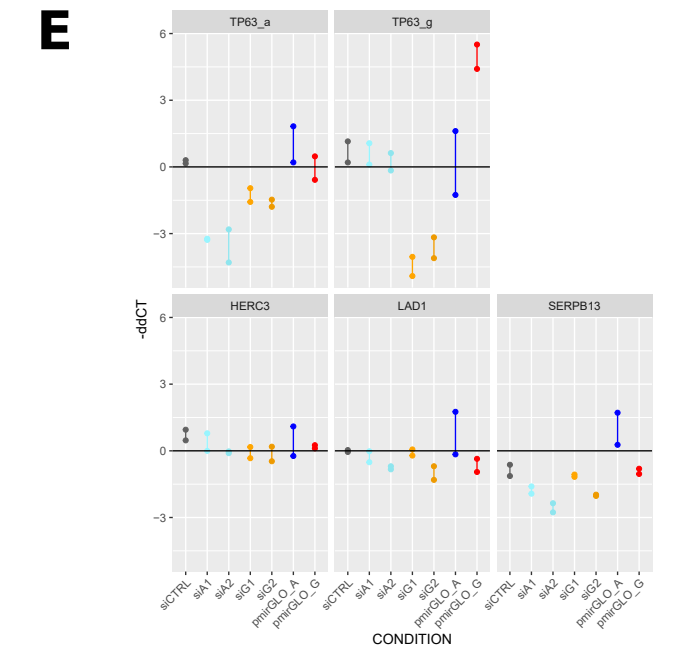
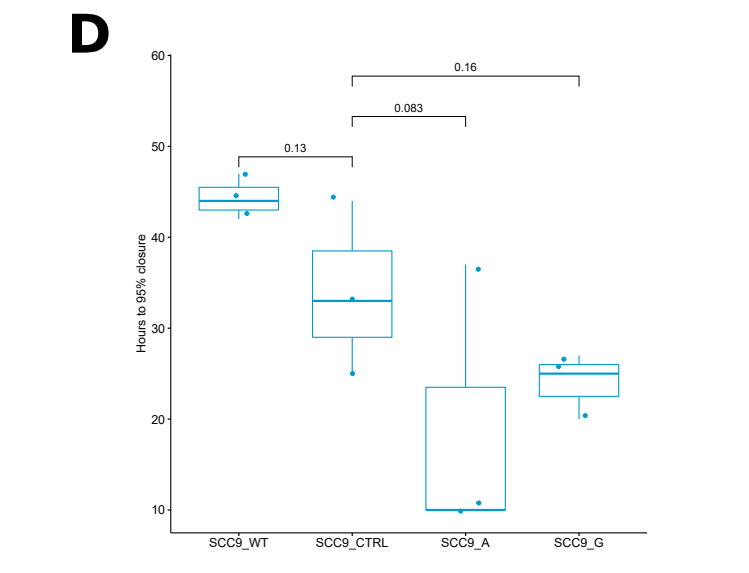
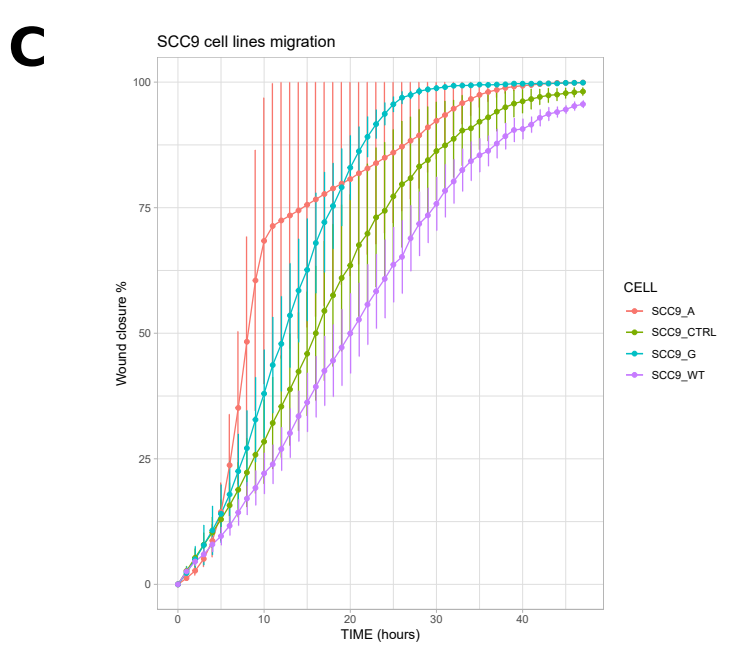
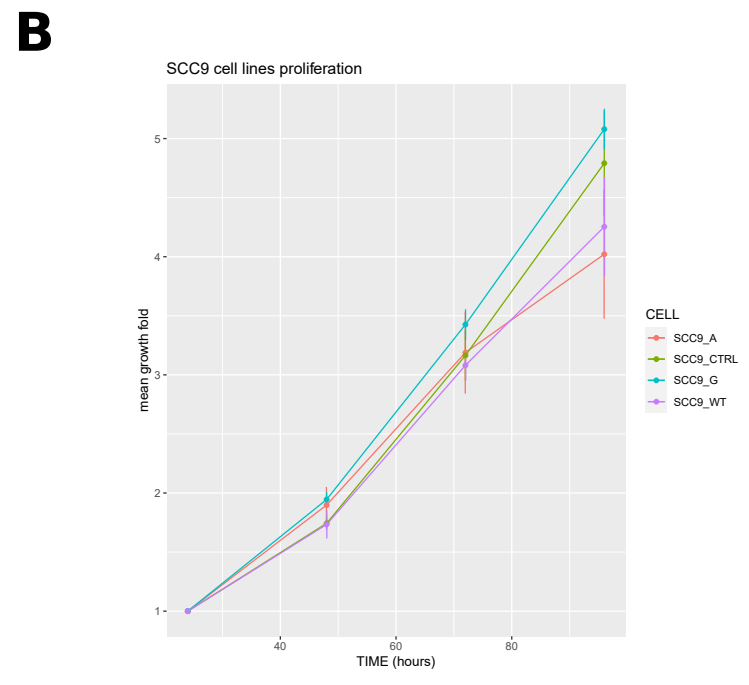
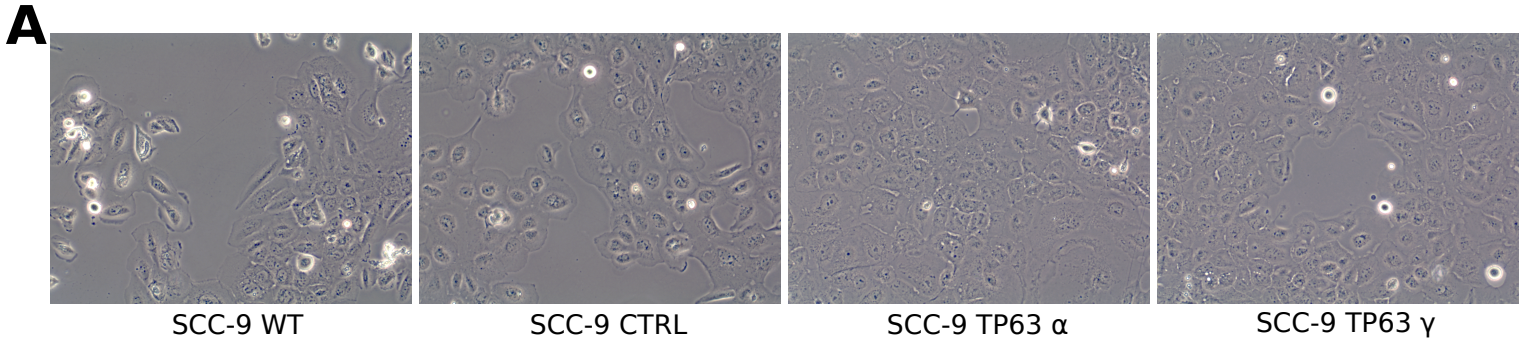


Figure.31. Phenotypic and molecular characterisation of SCC-9 multiclonal cell lines. **A.** Phase contrast microscopy images of subconfluent SCC-9 cells. WT: wild type, CTRL: cells expressing an empty pmirGLO GFP vector, *TP63 α* and *TP63 γ* : cells expressing *TP63 α* and γ from a pmirGLO GFP vector (see fig. 30) **B.** Cellular proliferation measured by automatic counting of DAPI stained nuclei on fixed cells at the indicated time after plating. **C.** Wound closure assays. Migration assessment was performed by time lapse microscopy on Ibidi cell culture inserts. Images were taken every hour. **D.** Comparison of time to 95% closure. **E.** Expression of *TP63* isoforms and selected previously described p63-target genes in overexpression cell lines or following 72h knock down of specific isoforms as indicated.

however in opposition with the negative regulation of *SERPINB13* proposed in (Riege et al., s. d.). Again, no effect of *TP63* γ modulation could be seen.

Overall, we have been unable to reproduce previously described effects of p63 on the EMT despite being able to drive EMT using TGF- β treatment. Surprisingly, we also struggled to find any significant changes in the expression of previously described p63 targets following isoform-specific depletion or up-regulation. To move forwards in the identification of p63 γ functions in HNSCC, clonal cell lines should be established overexpressing *TP63* α and γ . Rather than looking for individual targets, RNA sequencing of the different clones should be performed in order to identify their differentially expressed genes. Since there are currently no p63 γ -specific antibodies, expression of tagged p63 γ followed by Chip-seq could also be a good way of identifying its specific targets.

Chapter 3. Development of monoclonal antibodies targeting specific isoform of *TP63*

The absence of antibodies specific to the different C-terminal isoforms is a severe limitation in the study of both their functions and their pathological relevance. Isoform specific antibodies would allow the delineation of the expression domains of the different isoforms at the tissular, cellular or sub-cellular levels. From this, we could gain knowledge of the p63 containing complexes, which isoforms preferentially compose them, and where they are located.

The ability for health care practitioners to classify patients based on the expression of specific proteins in pathological samples allows them to adapt treatments to specific sub-populations of patients (Duraiyan et al. 2012; Lin et Chen 2014). Furthermore, in the case of proteins existing as multiple isoforms, immunological assessment of patient samples must be adapted to take into consideration the divergence in biological and clinical significance between these isoforms. As an example, isoform specific recognition of the 14-3-3 protein is used to distinguish sporadic Creutzfeldt-Jacob disease from other types of dementia (Everbroeck, Boons, et Cras 2005; Kuo et al. 2018). Isoform specific antibodies have also been useful for furthering our basic understanding of some diseases. In the case of Amyotrophic Lateral Sclerosis (ALS), C9orf72 isoform specific antibodies have identified differences in their subcellular localisations, which are modified in diseased cells (Smeyers, Banchi, et Latouche 2021; S. Xiao et al. 2015). In Alzheimer's disease and other Tauopathies, antibodies specific to the 0N, 1N and 2N Tau isoforms have enabled the identification of their respective partners, pointing towards a role of the 2N isoform in disease (C. Liu et al. 2016).

As we have shown that the specific overexpression of the *TP63 γ* isoform at the RNA level may be of prognostic value, it is important to determine whether this holds true at the protein level.

Isoform-specific antibodies are typically generated by using isoform-specific immunogenic peptides or recombinant proteins to promote antibody production (C. M. West et al. 2015). As stated earlier, multiple protein isoforms are encoded in the *TP63* gene (UniProtKB - Q9H3D4 (p63_HUMAN)) thanks to alternative splicing and alternative

promoter usage. While some polyclonal or monoclonal antibodies are available to detect some p63 isoforms (**Table 3**), their capacities to potentially cross-react with the other family members (p53 or p73) in addition to their capacity to detect multiple isoforms render their usage tricky in histology (Nekulova et al. 2013b). Indeed, the initial use of antibodies that could not distinguish between the different N-terminal isoforms of p63 made the analysis of p63 functions and expression quite complex in the early years. While numerous p63 specific antibodies are available, only few are isoform specific. Many target the DNA binding domain or the oligomerisation domain and are therefore able to detect all p63 protein isoforms and sometimes cross-react with the other family members (p53 or p73). Some are specific to the two N-terminal isoforms TA or Δ N and one, the D2K8X (Cell Signalling Technology) is specific to the C-terminal p63 α isoform. None are specific to the p63 γ or β isoforms. Therefore, we decided to try to develop novel p63 antibodies. Through a collaboration with the EFS (Etablissement Francais du Sang), we attempted to develop a panel of p63 antibodies that could prove useful in the study of p63 function or expression. Developing an antibody panel free of licensing rights could prove useful if we want to adopt a commercial strategy further down the line. To develop such p63 antibodies, we selected specific peptides located in precise domains of the p63 protein sequence and for which we could predict the protein isoforms that would be detected. Four peptides were designed to detect a) all Δ N p63 isoforms, b) all the p63 protein isoforms, c) the p63 gamma isoforms (Δ N or TA) and d) the p63 α isoforms (Δ N or TA) (**Figure 33.A**). We chose to avoid the p63 β or δ isoforms as only respectively 5 (RIWQV) or 7 (RSGKSENP) amino-acids are specific to those isoforms (see sequence **Figure 32**).

3.1. Mice immunisation and control

Peptides were ordered as biotin-labeled and Keyhole limpet hemocyanin (KLH)-labeled peptides from Protéogenix (SCHILTIGHEIM, France). Mice were immunized at the EFS using combinations of the chosen KLH-labeled peptides to increase the probability of a positive immune response. Because the absence of a p63 γ specific antibody is the most problematic, we chose to immunize mice using either a single gamma_p63_1 peptide (mice #112-115) or dual combinations of gamma specific peptide and of each of the other peptides: gamma + alpha (mice # 98-100 and # 102), gamma + Δ N (mice #101,#116-118), gamma + pan (mice #108-111). For each condition, four mice were challenged by 3 peptide injections.

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SP|Q9H3D4-2|P63_HUMAN MLYLENNAQTQFSEPQYTNLGLLSMDQQIQNGSSSTSPYNTDHAQNSVTAPSPYAQPSS 60
SP|Q9H3D4-4|P63_HUMAN MLYLENNAQTQFSEPQYTNLGLLSMDQQIQNGSSSTSPYNTDHAQNSVTAPSPYAQPSS 60
SP|Q9H3D4-6|P63_HUMAN MLYLENNAQTQFSEPQYTNLGLLSMDQQIQNGSSSTSPYNTDHAQNSVTAPSPYAQPSS 60
SP|Q9H3D4-8|P63_HUMAN MLYLENNAQTQFSEPQYTNLGLLSMDQQIQNGSSSTSPYNTDHAQNSVTAPSPYAQPSS 60
*****

SP|Q9H3D4-2|P63_HUMAN TFDALSPSPAIPSNTDYPGPHSFDVSFQSSTAKSATWYSTELKKLYCQIAKTCPIQIK 120
SP|Q9H3D4-4|P63_HUMAN TFDALSPSPAIPSNTDYPGPHSFDVSFQSSTAKSATWYSTELKKLYCQIAKTCPIQIK 120
SP|Q9H3D4-6|P63_HUMAN TFDALSPSPAIPSNTDYPGPHSFDVSFQSSTAKSATWYSTELKKLYCQIAKTCPIQIK 120
SP|Q9H3D4-8|P63_HUMAN TFDALSPSPAIPSNTDYPGPHSFDVSFQSSTAKSATWYSTELKKLYCQIAKTCPIQIK 120
*****

SP|Q9H3D4-2|P63_HUMAN VMTPPPQGAVIRAMPVYKKAEHVTEVVKRCPNHLSREFNEGQIAPPSHLIRVEGNNSHAQ 180
SP|Q9H3D4-4|P63_HUMAN VMTPPPQGAVIRAMPVYKKAEHVTEVVKRCPNHLSREFNEGQIAPPSHLIRVEGNNSHAQ 180
SP|Q9H3D4-6|P63_HUMAN VMTPPPQGAVIRAMPVYKKAEHVTEVVKRCPNHLSREFNEGQIAPPSHLIRVEGNNSHAQ 180
SP|Q9H3D4-8|P63_HUMAN VMTPPPQGAVIRAMPVYKKAEHVTEVVKRCPNHLSREFNEGQIAPPSHLIRVEGNNSHAQ 180
*****

SP|Q9H3D4-2|P63_HUMAN YVEDPITGRQSVLVPYEPPQVGTEFTTVLYNFMCSNSSCVGGMNRRPILIVTLETRDGQV 240
SP|Q9H3D4-4|P63_HUMAN YVEDPITGRQSVLVPYEPPQVGTEFTTVLYNFMCSNSSCVGGMNRRPILIVTLETRDGQV 240
SP|Q9H3D4-6|P63_HUMAN YVEDPITGRQSVLVPYEPPQVGTEFTTVLYNFMCSNSSCVGGMNRRPILIVTLETRDGQV 240
SP|Q9H3D4-8|P63_HUMAN YVEDPITGRQSVLVPYEPPQVGTEFTTVLYNFMCSNSSCVGGMNRRPILIVTLETRDGQV 240
*****

SP|Q9H3D4-2|P63_HUMAN LGRRCFEARICACPRDRKAEDSIRKQVSDSTKNGDGTKRPFRQNTHGIQMTSIKKRR 300
SP|Q9H3D4-4|P63_HUMAN LGRRCFEARICACPRDRKAEDSIRKQVSDSTKNGDGTKRPFRQNTHGIQMTSIKKRR 300
SP|Q9H3D4-6|P63_HUMAN LGRRCFEARICACPRDRKAEDSIRKQVSDSTKNGDGTKRPFRQNTHGIQMTSIKKRR 300
SP|Q9H3D4-8|P63_HUMAN LGRRCFEARICACPRDRKAEDSIRKQVSDSTKNGDGTKRPFRQNTHGIQMTSIKKRR 300
*****

SP|Q9H3D4-2|P63_HUMAN SPDDELLYLPVRGRETYEMLLKIKESLELMQYLPQHTIETYRQQQQQHQHLLQKQTSIQ 360
SP|Q9H3D4-4|P63_HUMAN SPDDELLYLPVRGRETYEMLLKIKESLELMQYLPQHTIETYRQQQQQHQHLLQKQTSIQ 360
SP|Q9H3D4-6|P63_HUMAN SPDDELLYLPVRGRETYEMLLKIKESLELMQYLPQHTIETYRQQQQQHQHLLQKQTSIQ 360
SP|Q9H3D4-8|P63_HUMAN SPDDELLYLPVRGRETYEMLLKIKESLELMQYLPQHTIETYRQQQQQHQHLLQKQTSIQ 360
*****;

SP|Q9H3D4-2|P63_HUMAN SP-----SSYGNSSPPLNKMNSMNKLPSVSQLINPQQRNALPTTIPDG 404
SP|Q9H3D4-4|P63_HUMAN SP-----SSYGNSSPPLNKMNSMNKLPSVSQLINPQQRNALPTTIPDG 404
SP|Q9H3D4-6|P63_HUMAN CFRNELVEPRPRETPKQSDVFFRHSKPPNRSVY----- 393
SP|Q9H3D4-8|P63_HUMAN SP-----SSYGNSSPPLNKMNSMNKLPSVSQLINPQQRNALPTTIPDG 404
. :*.** ..

SP|Q9H3D4-2|P63_HUMAN MGANIPMMGTHMPMAGDMNGLSPTQALPPPLSMPSTSHCTPPPPYPTDCSIVSFLARLGC 464
SP|Q9H3D4-4|P63_HUMAN MGANIPMMGTHMPMAGDMNGLSPTQALPPPLSMPSTSHCTPPPPYPTDCSIVRIWQV--- 461
SP|Q9H3D4-6|P63_HUMAN -----
SP|Q9H3D4-8|P63_HUMAN MGANRSGKSENP----- 416

SP|Q9H3D4-2|P63_HUMAN SSCLDYFTTQGLTTIYQIEHYSMDDLASLKIPEQFRHAIWKGLDHRQLHEFSSPSHLLR 524
SP|Q9H3D4-4|P63_HUMAN -----
SP|Q9H3D4-6|P63_HUMAN -----
SP|Q9H3D4-8|P63_HUMAN -----

SP|Q9H3D4-2|P63_HUMAN TPSSASTVSVGSSETRGERVIDAVRFTLRQTISFPPRDEWDFNDFMDARRNKQQRKEE 584
SP|Q9H3D4-4|P63_HUMAN -----
SP|Q9H3D4-6|P63_HUMAN -----
SP|Q9H3D4-8|P63_HUMAN -----

SP|Q9H3D4-2|P63_HUMAN GE 586
SP|Q9H3D4-4|P63_HUMAN --
SP|Q9H3D4-6|P63_HUMAN --
SP|Q9H3D4-8|P63_HUMAN --

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 DNA BINDING DOMAIN

 TETRAMERIZATION DOMAIN

 STERILE ALPHA MOTIF

Figure 32: Protein sequence and peptides for the different p63 isoforms.

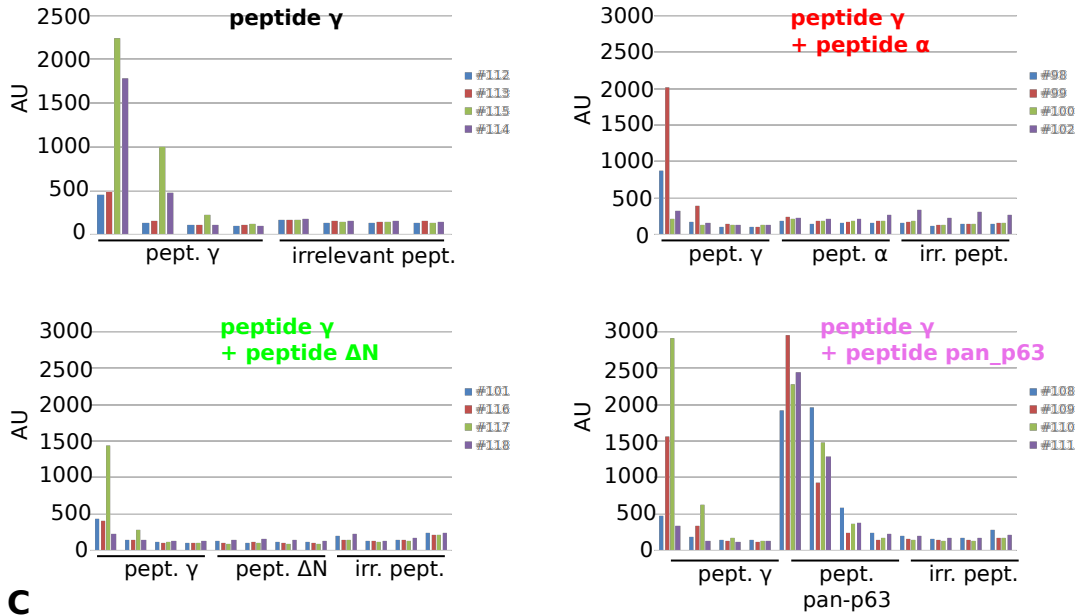
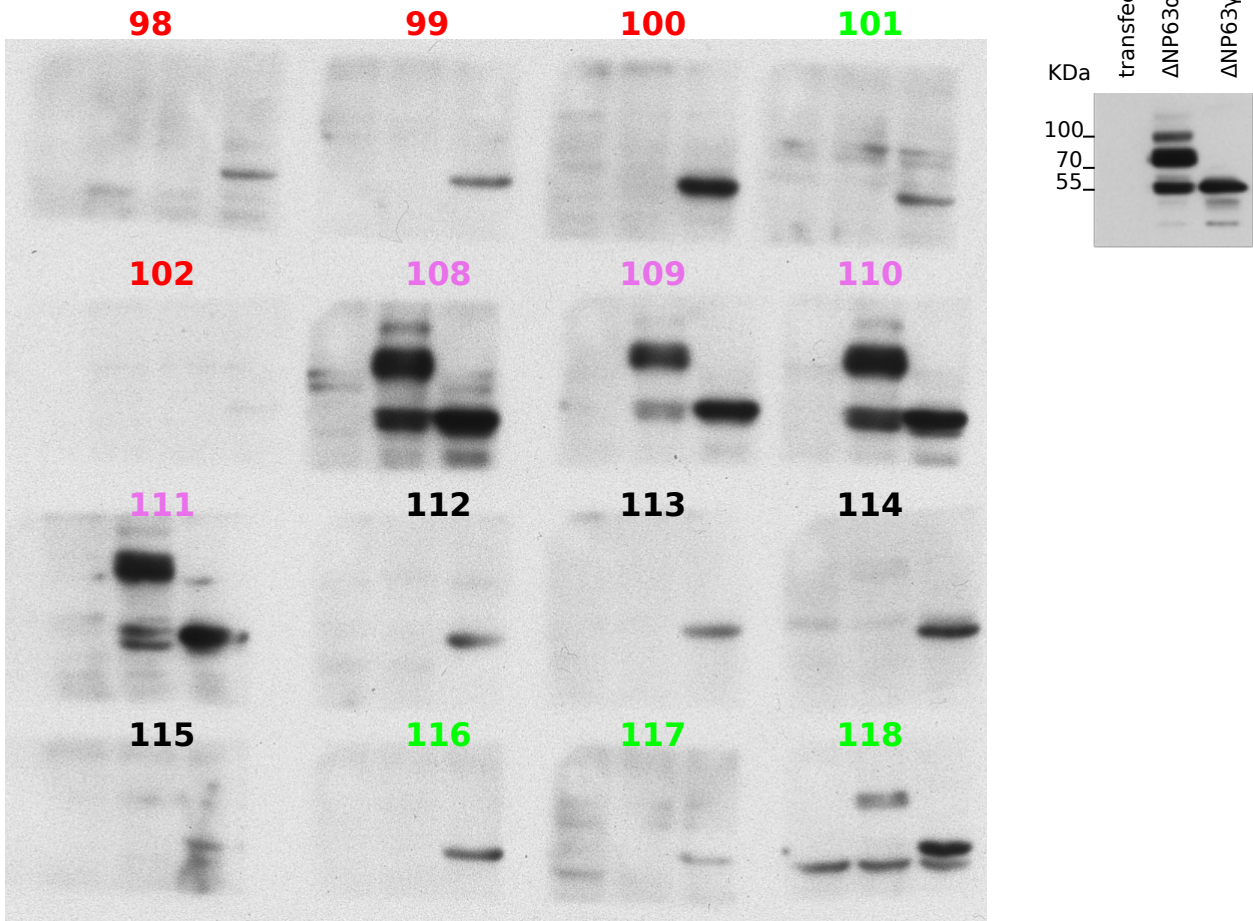
Only ΔN isoforms are shown . Q9H3D4-2: ΔNp63 alpha, Q9H3D4-4: ΔNp63 beta, Q9H3D4-6: ΔNp63 gamma, Q9H3D4-8: ΔNp63 delta. The protein domains are identified using PFAM and the sequences are highlighted accordingly. The peptides used in the immunisation process are in bold. The epitope for the 4A4 monoclonal antibody is underlined

A

ΔN	MLYLENNAQTQFSE
pan_p63	SHAQYVEDPITGRQSVLPY
γ	CFRNELVEPRRET
α	ERVIDAVRFTLRQTISF

a) Biotin-conjugated

b) KLH-conjugated

B**C****Figure 33: Immunisation and selection**

A. The four peptides specific for the indicated TP63 isoforms are shown. They were ordered conjugated to biotin (for Elisa) or to Keyhole limpet hemocyanin (KLH) for immunisation.

B. Elisa analysis of mice sera after 3 rounds of immunisation with the indicated peptide combinations (on top of the graph). Four sera dilutions were tested for each mouse on an irrelevant peptide or p63 peptides. Mice IDs are indicated on the right of each graph. Signal is represented as arbitrary units (AU).

C. Western-blot analysis of HeLa cellular extract expressing recombinant $\Delta Np63\alpha$ proteins, recombinant $\Delta NP63\gamma$ proteins or none, as indicated. Western-blot was conducted with diluted sera (1/10th in TBST 5% milk) for each of the mice tested in B. Right upper panel: membrane was analysed using a commercial p63 antibody for comparison (4A4). Molecular weights are indicated on this test membrane.

Table 3: most common antibodies used in TP63 studies

reference	provider	immunogen	description/ specificity	species	Epitope mapping
DAK-p63 (GA662)	DAKO	synthetic peptide derived from the core DNA-binding domain of the human p63	ALL p63 isoforms	Mice mAb	
BC28	Abcam	synthetic peptide corresponding to Δ Np63 amino acids 5-17 (ENNAQTQFSEPQY)	Only Δ N isoforms	Mice mAb	
4A4	Abcam	Recombinant fragment corresponding to human Δ Np63 (AA 1-205)	Only Δ N isoforms	Mice MAb	IPSN (Nekulova et al. 2013)
D2K8X	Cell signalling technology	Synthetic peptide corresponding to residues near the carboxy terminus of human p63- α protein	p63 α only isoforms	Rabbit mAb	

After the third injection, small aliquots of blood were taken from mice for analysis. Elisa was conducted with serum dilutions against baits (p63 peptides) and control peptides to assess the development of an immune response against the peptides and determine the nature of the peptide targeted when combinations were used (**Figure 33.B**). Mice 114 and 115 developed a response against peptide γ . Mice 98 and 99 developed a response against peptide γ but not to peptide α . Mice 117 developed a response to peptide γ and not to peptide ΔN . Mice 109 and 110 developed responses to peptides γ and to peptide Pan-p63; and mice 108 and 111 developed responses to peptide Pan-p63 only. Therefore the immune responses appeared to be limited to peptides γ and Pan-p63. No response to peptides ΔN or α could be observed.

To further assess the ability of the sera to specifically detect the p63 isoforms of interest, we tested them by western-blot analysis using recombinant proteins. HeLa cells do not express p63 or only at very low levels. As such they are a suitable model to assess the capacity of the newly generated antibodies to detect recombinant p63 protein, with low endogenous targets which could confuse the results. As shown on **Figure 33.C**-right panel, HeLa cells were transfected with plasmid constructs producing $\Delta Np63\alpha$ and $\Delta Np63\gamma$ proteins, and recombinant proteins were detected using the 4A4 commercial antibody that detects all peptide isoforms.

Using the 4A4 antibody, $\Delta NP63\gamma$ is detected as a single band at 55 Kda for a predicted size of 44,7KDa. $\Delta NP63\alpha$ is detected as a major band slightly above 70 KDa (predicted size 65,8 Kda) with a lighter signal at 55KDa which could be a degradation product. No signals could be detected in the transfectant only control. We used multiple replicates of this membrane to assess the different immune sera (**Figure 33.C**). All mice challenged with the γ peptide, except mouse 112, could detect the $\Delta NP63\gamma$ recombinant protein with differing intensity and background signals. Mice challenged with the α peptide (98, 99, 100, 102) could not detect the $\Delta NP63\alpha$ recombinant protein. Similarly, mice challenged with the ΔN peptide (101, 116-118) could not detect the $\Delta NP63\alpha$ recombinant protein. Both of these results being coherent with the negative Elisa results for the sera from these mice. Mice (108-111) challenged with the Pan-p63 peptides could all detect the $\Delta NP63\alpha$ recombinant proteins. It is noteworthy that antibodies directed to the pan-p63 peptide would also cross react with the $\Delta NP63\gamma$ recombinant protein. This is probably why among the different sera evaluated on $\Delta NP63\gamma$ proteins, mice sera (108-111) gave the strongest $\Delta NP63\gamma$ signal.

To try to improve the sera's antibody titer and try to develop antibodies to α and ΔN peptides, the same mice were further challenged by two sets of injections with γ peptide, α peptide or ΔN peptide. Sera titer was controlled by Elisa (**Figure 34**). Elisa results confirmed the absence of immunisation against the ΔN and the α peptides and highlighted an increase in the titer of γ specific antibodies.

Based on these results we proceeded to select mice that would be used for fusion of B cells with myeloma cells to generate antibody producing hybridomas.

3.2. Hybridoma generation

To choose which mice were going to serve for hybridoma productions, the main criteria were:

- a) a good correlation between Elisa results and Western-blotting results,
- b) a strong detection of the recombinant protein in Western-Blot and of its peptide in Elisa,
- c) a low background in Western-blot

Mouse 110, that was immunized against both the γ peptide and the Pan-peptide gave a strong signal in ELISA for both the γ peptide and the pan peptide. In western-blot serum from mice 110 could clearly detect both the $\Delta Np63\gamma$ and the $\Delta Np63\alpha$ recombinant proteins indicating that antibodies targeting both peptides were produced by the mouse. This mouse was chosen for hybridoma development. Following fusion and selection, out of 1935 growing clones we recovered 8 different hybridoma clones that tested positive in Elisa for specific recognition of either pan-p63 peptide (5 clones: KO382, KO561, KO1133, KO1393, KO1791) or the γ peptide (3 clones: KO85, KO474, KO1356) (**Table 4**). We then tested these 8 clones by western-blot on recombinant protein as previously done for the sera (**Figure 35**). Among the γ specific clones, only the clone KO85 rendered a strong positive and specific signal to $\Delta Np63\gamma$ in western-blot. Among the Pan-specific clones, the results were somewhat unexpected. Only clone KO1393 gave a strong signal with both the $\Delta NP63\alpha$ and $\Delta NP63\gamma$ recombinant proteins. The KO1393 pan-p63 clone also recognises a protein of similar molecular weight to p63 γ in the p63 α lane. This band is not recognised by the clone KO85 specific to p63 γ , suggesting that this is most likely a degradation product of p63 α . Among the three other pan-p63 clones, only signals from the $\Delta NP63\gamma$ recombinant protein could be detected at a somewhat low intensity level. No detection of the $\Delta NP63\alpha$ could be observed.

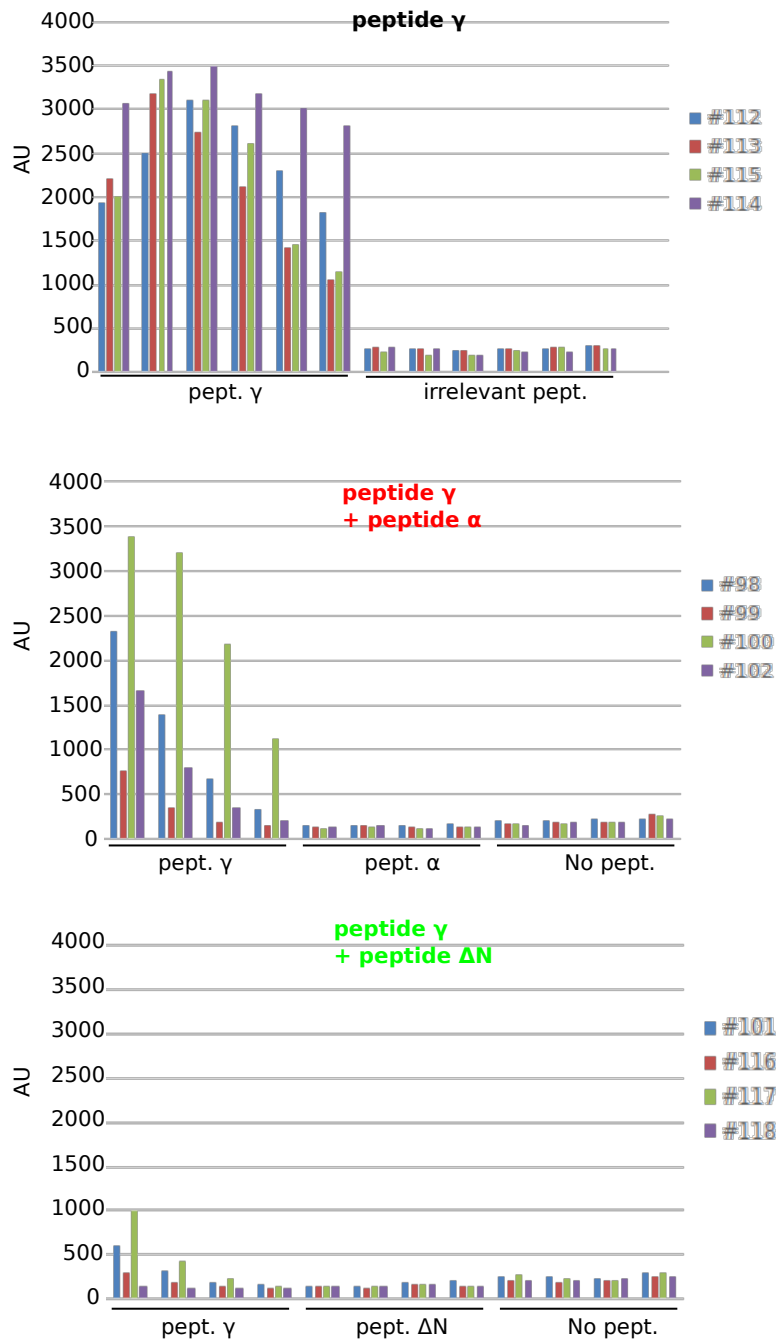


Figure 34: Second round of immunisation and selection.

The selected mice were challenged two more times with the indicated peptides. Elisa analysis of mice sera after 3 rounds of immunization with the indicated peptide combinations (on top of the graph). 4 (or 6 upper panel) sera dilutions were tested for each mouse on an irrelevant peptide or on the indicated p63 peptides. Mice IDs are indicated on the right of each graph. Signal is represented as arbitrary units (AU).

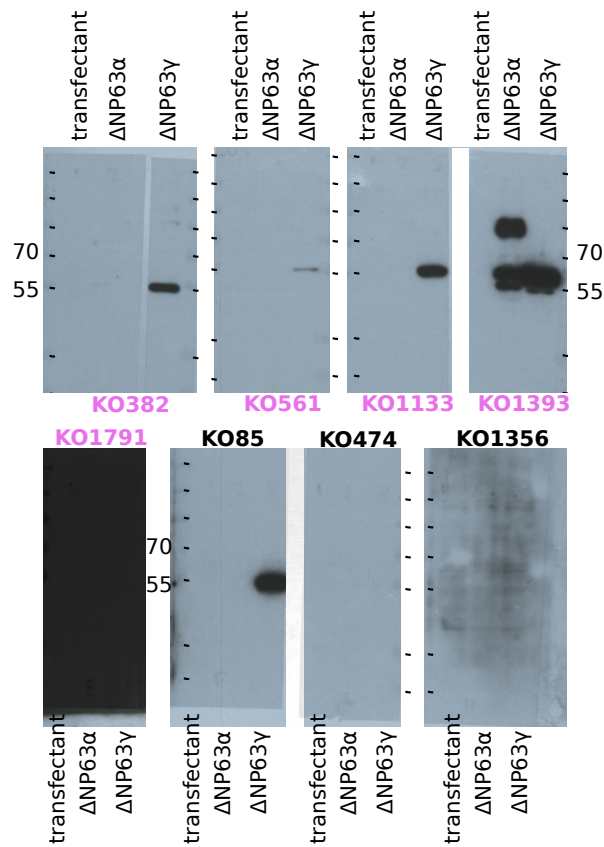


Figure 35: Testing by Western-blot of ELISA positive hybridoma clones

Western-blot analysis of HeLa cellular extract expressing none, recombinant Δ NP63 α or recombinant Δ NP63 γ proteins as indicated. Western-blot was conducted with the indicated hybridoma supernatants. Pink/pan-p63 positive hybridoma, black/ γ -peptide positive hybridoma.

Table 4: Hybridoma development and selection

Fusion hybridomas were selected, amplified and tested for immunoreactivity to Pan or γ peptides. .

HYBRIDOMA SELECTIONS		
seeded wells	2880	
tested wells	1935	67,00 %
positive wells	70	4,00 %
clone amplified in 24-wells	70	100,00 %
amplified clones tested positive	13	19,00 %
frozen positive clones	8	61,00 %

3.3. Assessing the sensitivity and specificity of the purified antibodies

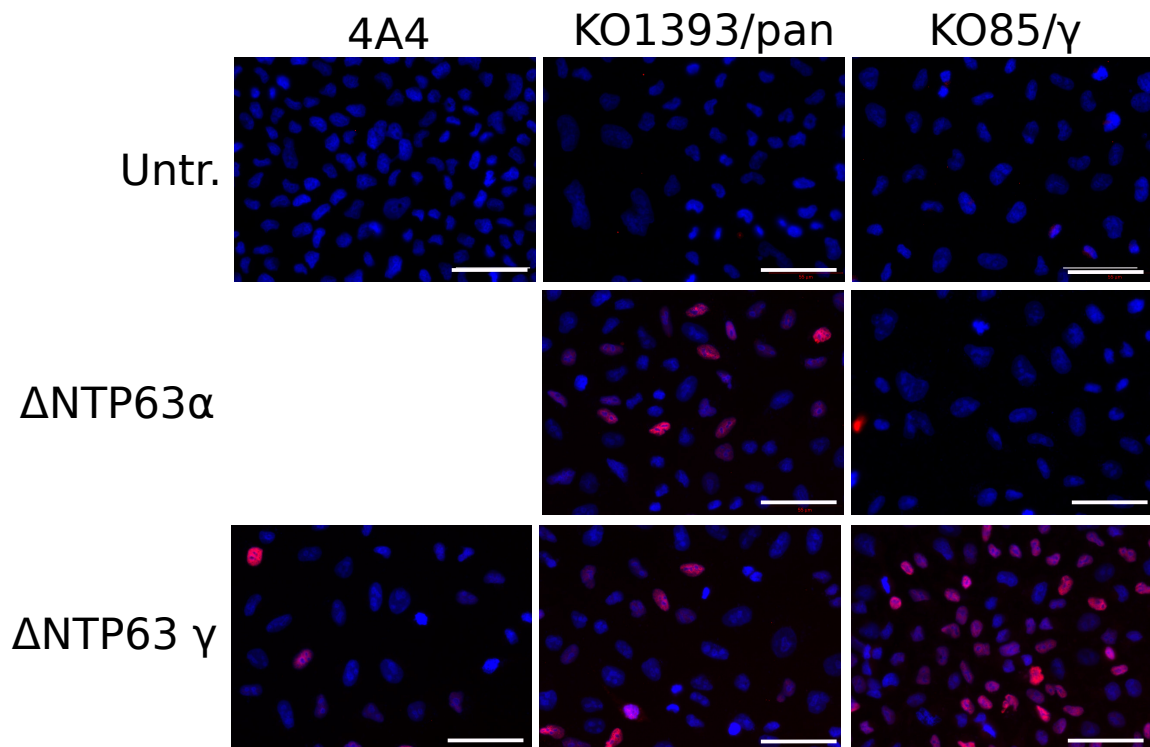
For further analysis we chose to focus on the KO1393/pan *TP63* and KO85/ γ -specific clones. We wanted to determine the sensitivity and specificity of both clones. Immunoglobulins from the hybridoma clone supernatants were purified on A/G proteins and concentrated. For the following experiments we exclusively used the purified antibodies.

To determine if the antibodies could be used to detect endogenous or recombinant protein by immunofluorescence, we used two distinct cell lines transfected with plasmids expressing p63 α , β or γ isoforms. HeLa cells were once again used, taking advantage of their lack of endogenous p63, and SCC-9 cells were used as an easy to transfect p63 positive cell line. In practice, neither cell line showed endogenous staining of p63 by IF, even using the 4A4 commercial antibody. Transfection of recombinant p63 proteins allowed us to achieve sufficient p63 levels to observe IF signals. In both cell lines, antibodies from the KO1393/pan clone specifically stained nuclei expressing p63 isoforms. Likewise, antibodies from the KO85/ γ -specific clone only stained nuclei from cells transfected with $\Delta NTP63\gamma$ vectors. (see **Figure 36**). This indicated that both the pan and γ -specific clones could detect overexpressed p63 in immunofluorescence experiments, and the γ -specific clone could do this in an isoform-specific manner.

Assessing the capacity of the novel antibodies to recognise endogenous p63 in the immunofluorescence context will require the use of cell lines expressing higher p63 levels, or increasing the titer of the antibodies.

In parallel, we tested the specificity and sensitivity of the new p63 antibodies for the recognition of recombinant or endogenous p63 protein using western-blot. First, we tested the KO1393/pan and KO85/ γ -specific antibodies on HeLa cell extracts overexpressing the three different p63 isoforms α , β and γ as previously described (**Figure 37.A**). The KO85 antibody (**Figure 37.A**, upper panel) recognised recombinant p63 γ with very little non-specific signal, however the exposure time required for signal detection is quite high (31,4 s). The KO1393 purified antibody (**Figure 37.A**, middle panel) identified protein bands corresponding to p63 α , β or γ depending on the overexpressed proteins, and several protein species below the 55 KDa marker which could be degradation products of the expressed recombinant protein. As previously seen **figure 35**, it recognised at 55KDa a species that is not identified by the KO85 antibody. The signals obtained were highly

A HeLa



B SCC9

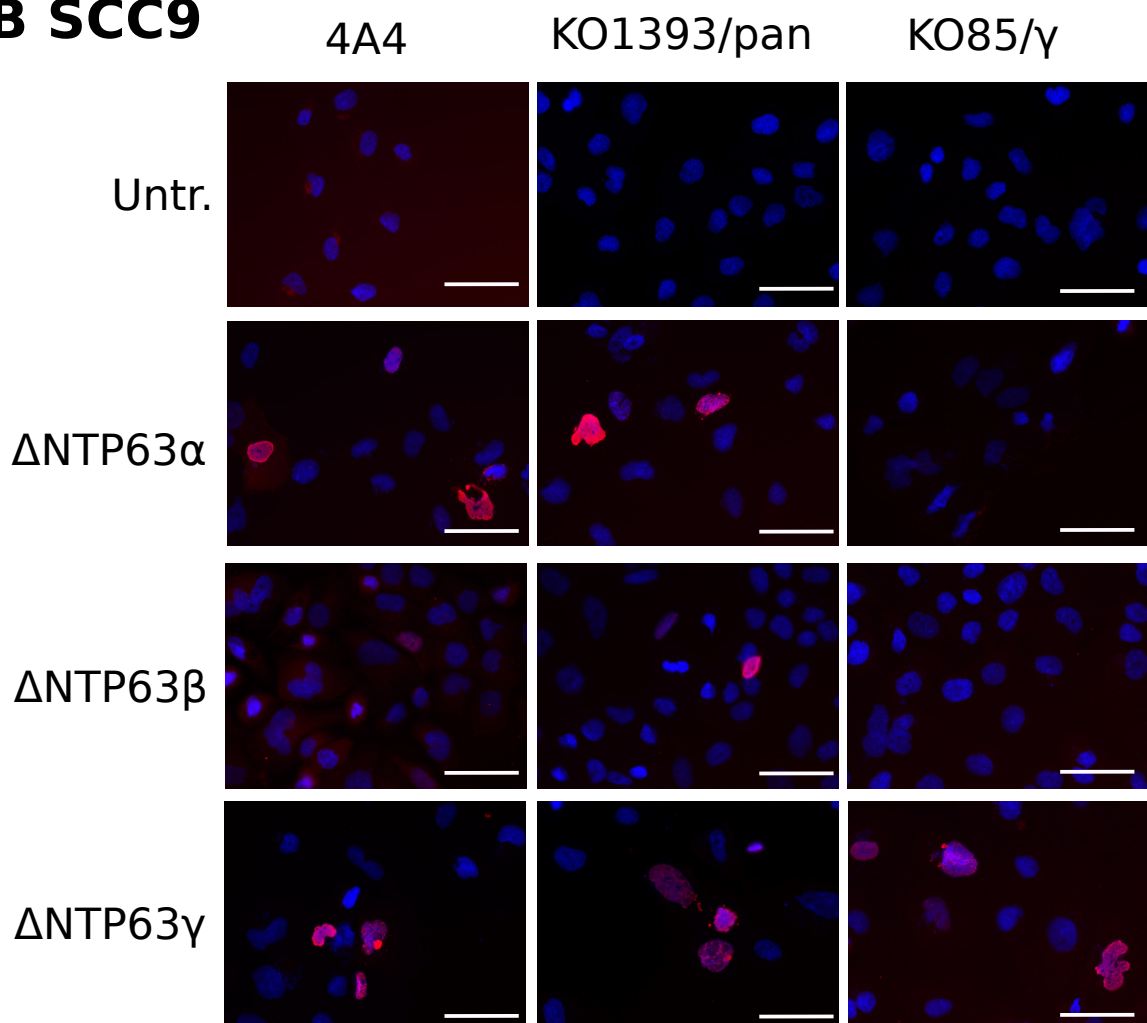


Figure 36. Immunofluorescence analysis of the specificity of KO1393/pan and KO85/ γ p63 antibodies.

HeLa cells (**A**) or SCC-9 cells (**B**) were transfected with the indicated pCDNA3.1 vectors for 48h prior to PFA fixation and staining with the indicated antibodies. Nuclei were stained with DAPI. Images are representative of the staining obtained in each condition.

In HeLa cells expressing Δ Np63 α stained with 4A4 antibody, insufficient cells had attached to the glass coverslip making this condition unusable.

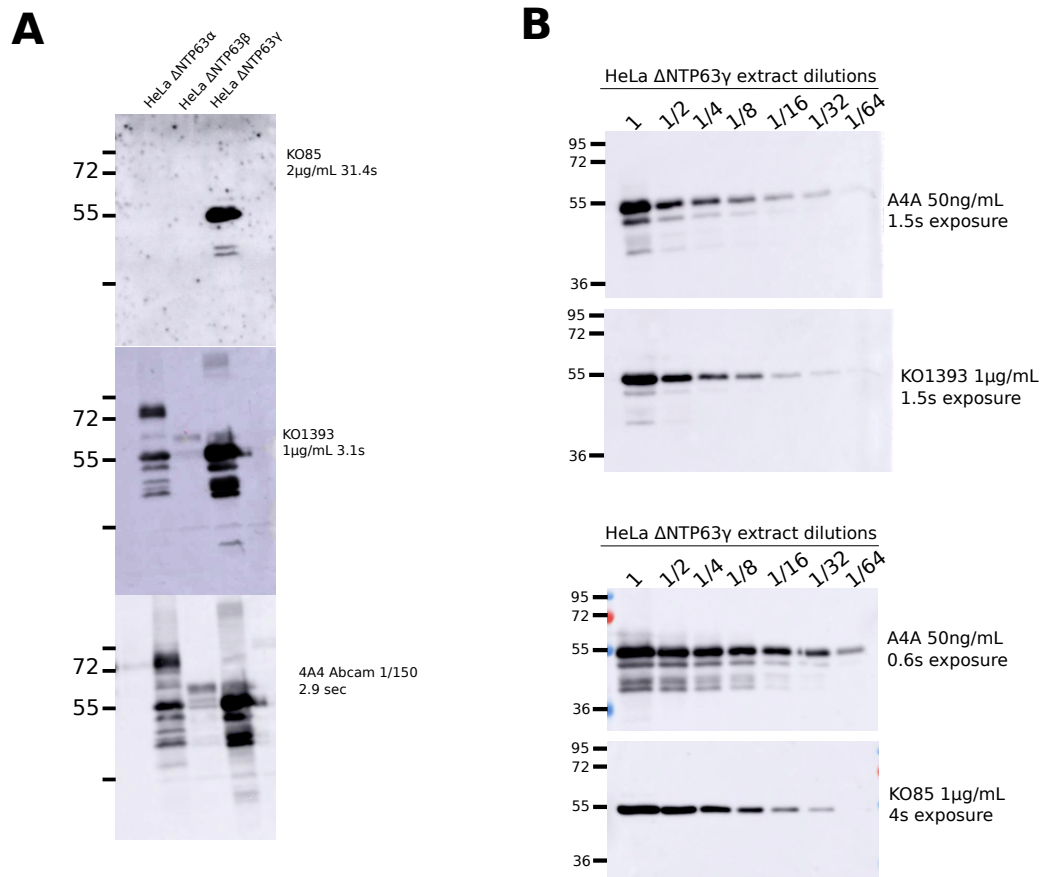


Figure 37: Detection of overexpressed p63 α β and γ isoforms using commercial pan-p63 (4A4), or using pan-p63 antibody (KO1393) and γ -specific antibody (KO85) we developed.

A. HeLa cells were transfected with Δ Np63 α , β , or γ expressing plasmids (under the control of a pCMV promoter). Cell extracts were size fractionated and p63 protein detected using the indicated antibodies. **B.** Serial dilutions of extract from HeLa cells transfected with Δ Np63 γ expressing plasmids. The size fractionated proteins were detected using either the 4A4, the KO1393 or the KO85 antibodies. Total cellular extract was used for western blotting. Molecular size markers are shown on the left side of the membranes.

similar to WB signals obtained with the 4A4 monoclonal antibody (**Figure 37.A**, lower panel)

We compared the sensitivity of the KO1393 and KO85 antibodies to the 4A4 commercial pan-p63 antibody, by performing serial dilutions of HeLa cell extracts expressing p63 α or γ on duplicate membranes (**Figure 37.B**). The KO1393 was only slightly less sensitive than the 4A4 antibody, with similar detection time and roughly similar signal detection (compare the two upper panels). However the sensitivity of the KO85 monoclonal purified antibody was clearly lower than that of the 4A4 with longer required exposure time and absence of detection in the 1/64 dilution sample. Nonetheless, in the context of the detection of recombinant p63 protein, the specificity of these antibodies seemed excellent, especially the γ -specific KO85.

These extracts used so far came from cells overexpressing p63 isoforms at high levels, driven by a CMV promoter. We therefore sought to test if the KO85 antibodies could detect p63 γ specifically and with enough sensitivity on cells with lower overexpression of p63. For this we used a second set of p63 expressing vectors derived from pmirGLO, with a PGK promoter driving their expression.

Transfection of the pmirGLO *TP63* γ expressing plasmid into SCC-9 or A253 cells that normally express p63 was chosen to allow for an estimation of the background detection in a more relevant cell line than HeLa (**Figure 38**). Multiclonal SCC-9 and A253 cell lines were obtained following these transfections and used for analysis. Extract from HeLa cells expressing p63 γ was used as a positive control. In SCC-9 extracts (**Figure 38.A**), the 4A4 antibody recognised a band (*) slightly below the 55 KDa marker in SCC9 + pmirGLO *TP63* γ . This band likely corresponds to p63 γ isoform, as it is undetected in SCC9 + pmirGLO GFP_ctrl and it is not recognized by the D2K8X antibody. The transfection of pmirGLO *TP63* γ in A253 cells did not allow for detection of the p63 γ isoform. The KO85 antibody only seemed to specifically recognise the p63 γ present in abundance in the HeLa extract, with no signal at the correct height in the SCC9 + pmirGLO *TP63* γ lane. Because of long exposure time, high background staining was accumulating. The same conclusion could be made about the KO1393 antibody, but with far less background noise. All in all, this experiment indicates that despite specific recognition of their target epitopes when present in high concentration, the KO1393 and KO85 lack sensitivity to detect targets at lower concentration.

To further test the specificity and sensitivity of the KO85 antibody, we performed serial dilutions of HeLa p63 γ extracts in SCC-9 cell extracts. This would provide us with

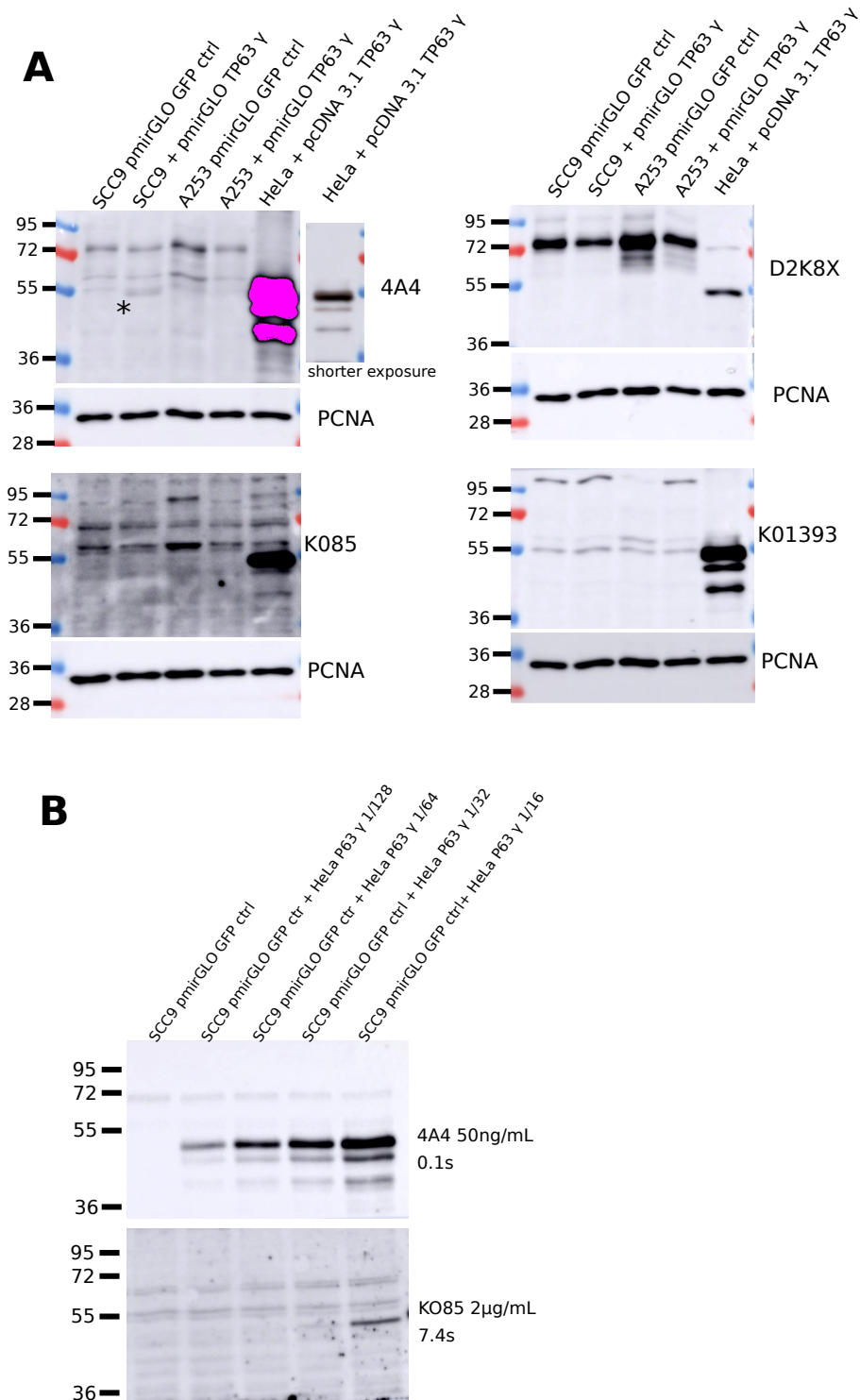


Figure 38: Detection of endogenous and ectopic p63 isoforms using commercial pan-p63 (4A4), p63 α specific (D2K8X) antibodies, or using pan-p63 (KO1393) and γ -specific (KO85) antibodies we developed. **A.** SCC-9 or A253 cells were transfected with control (pmirGLO GFP ctrl) or p63 γ expressing plasmids (pmirGLO TP63 γ). Stable multiclonal lines were obtained by antibiotic selection. HeLa cells expressing TP63 γ under the control of a strong pCMV promoter serve as a positive control. PCNA is used as a loading control. **B.** SCC9 extract was mixed with a serial dilution of HeLa cell extract expressing TP63 γ . The size fractionated proteins were detected using either the 4A4 or the KO85 antibodies. Total cellular extract was used for western blotting.

both detectable signal levels, and an experimentally pertinent context for appreciation of background noise. The same samples were deposited in duplicate series to compare the 4A4 and KO85 antibodies (**Figure 38**). On SCC9 extracts, the 4A4 antibody recognised a protein just below 72 KDa, coherent with the detection of the endogenous p63 α protein from SCC9 cells. In SCC9 cells mixed with a serial dilution of HeLa cell extract expressing p63 γ , the 4A4 antibody detected a p63 γ band and its likely degradation products specifically even at the strongest dilution (1/128). Comparatively, the KO85 only provided signal superior to background noise at the highest concentration of HeLa extract (1/16 dilution) and with an exposure 74 times longer. Several non-specific species were also recognised by this antibody with such a long exposure. From this experiment, we could estimate that the KO85 purified antibody is about 600 times less sensitive than the 4A4 antibody. Part of the background noise problem is likely down to the poorer sensitivity of the KO85 antibody compared to the 4A4 antibody, which forces the use of longer exposure times.

SCC-25 cells express high levels of p63 α , and were therefore chosen to test the recognition of endogenous p63 by the KO1393/pan antibody and compare it to the commercial D2K8X and 4A4 antibodies (**Figure 39**). To further assess the specificity of the antibodies for p63 proteins, we depleted the SCC-25 cells by siRNA directed against all *TP63* isoforms, or against *TP63 α* specifically. A strong signal at 72 KDa is observed with the 4A4 and D2K8X antibodies and correspond to the p63 α isoform as confirmed by the vast decrease in this signal intensity upon treatment with siPan and siA1 or siA2. The KO1393/pan antibody was capable of recognising the same endogenous p63 α band with a lesser intensity than the commercial antibodies. The KO1393/pan antibody detected significantly more non-specific bands than both the D2K8X and the 4A4 antibodies. Furthermore, the p63 α band recognised by the KO1393/pan antibody is much weaker than several of its non-specific targets. This raises serious concern over its potential use in immunofluorescence based applications where specificity is crucial.

3.4. Conclusion

Despite having developed novel monoclonal antibodies capable of detecting either all p63 isoforms or specifically the p63 γ isoform when present at high concentrations, we have fallen short of achieving usable results when dealing with the endogenously low p63

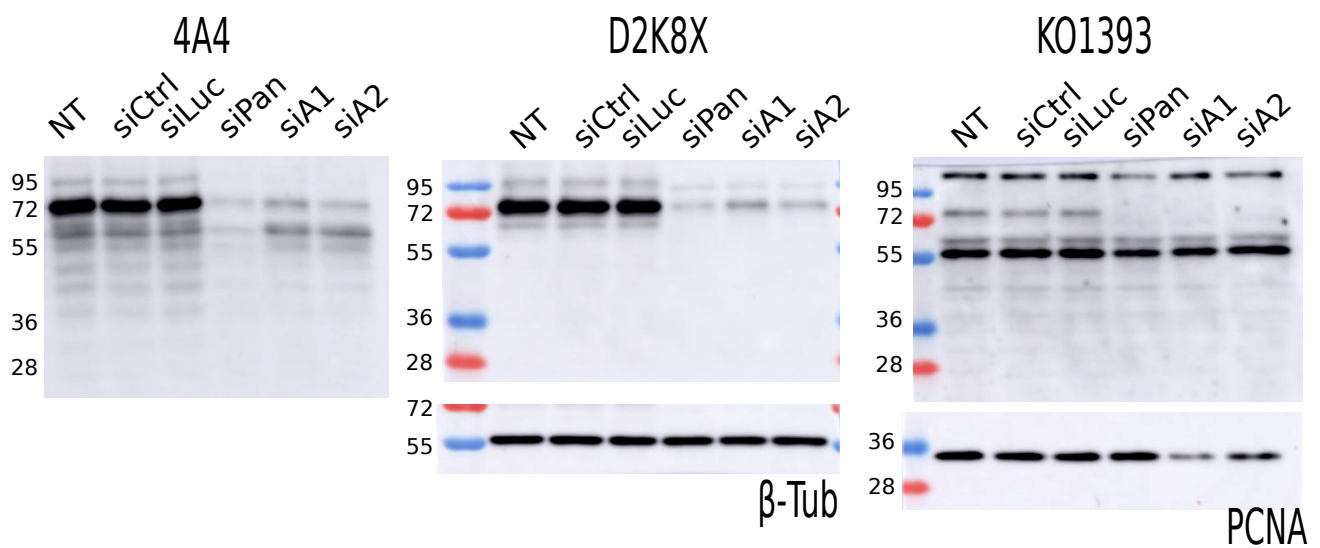


Figure 39: Detection of endogenous p63 comparing commercial pan-p63 (4A4) commercial p63 α specific (D2K8X) antibodies, or pan-p63 antibodies (KO1393). SCC-25 cells were depleted in all p63 isoforms (siPan), specifically depleted in p63 α (siA1 and siA2) or treated with control siRNAs (siCtrl and siLuc). NT cells received only transfectant. Total cellular extract was used for western blotting with the indicated antibodies .

Chapter 4. Identification of circRNAs derived from the *TP63* gene in head and neck squamous cell carcinoma (HNSCC)

Since the initial discovery of circular RNA from viroids in the late 70s and the description of some singular cases of eukaryotic circular RNA in the 90s, the development of high throughput sequencing techniques has generalised the existence of such circular RNA (Patop, Wüst, et Kadener 2019).

By an alternative pathway, pre-mRNAs can produce circulars RNA (circRNA). This process termed back-splicing is a splicing reaction where a donor site (SD) at the 3'end of an exon is spliced to an upstream acceptor site (SA), which can be in the same exon or in a previous exon. The resulting phosphodiester bond creates a looped molecule: the circRNA (**Figure 40**). Over 25 000 circular RNAs, derived from around 15% of actively transcribed genes, were identified from a single cell type (Jeck et al. 2013). Backsplicing of exons for circRNA formation can be considered as an unusual type of alternative splicing. Back-splicing, however, represents less than 1% of canonical splicing in most endogenous human loci (Yang Zhang et al. 2016). The usage of splice sites is competitive between back-splicing and canonical splicing. Increasing back-splicing can therefore increase the proportion of linear RNAs lacking the circularized exons. When abundance of core spliceosomal factors becomes limited, it has been observed that back-splicing appears to be more efficient than the production of linear mRNA. This likely results from remodeling of cross-intron exon definition complexes (D. Liang et al. 2017). Indeed, exon definition complexes can be deposited on single exons, which become catalytically competent for back-splicing reactions (Xueni Li et al. 2019). This back-splicing event requires the donor and acceptor sites to be in close proximity to each other. This is made possible either by the presence of Inverted Complementary Sequences (ICS), such as *Alu* sequences, in the introns flanking circRNA-exons . Repetitive elements are enriched in the human genome, but in other organisms, ICS appear to be less crucial. As an illustration, production of circRNA in *D. melanogaster* is generated from exons with long flanking introns lacking ICS (Yang Zhang et al. 2016). Besides repeated elements, a number of RBPs that bind the flanking introns can be involved in circRNA biogenesis. By directly bridging distal splice sites, or by binding ICS to modulate back-splicing, RBPs can either stabilize the transiently

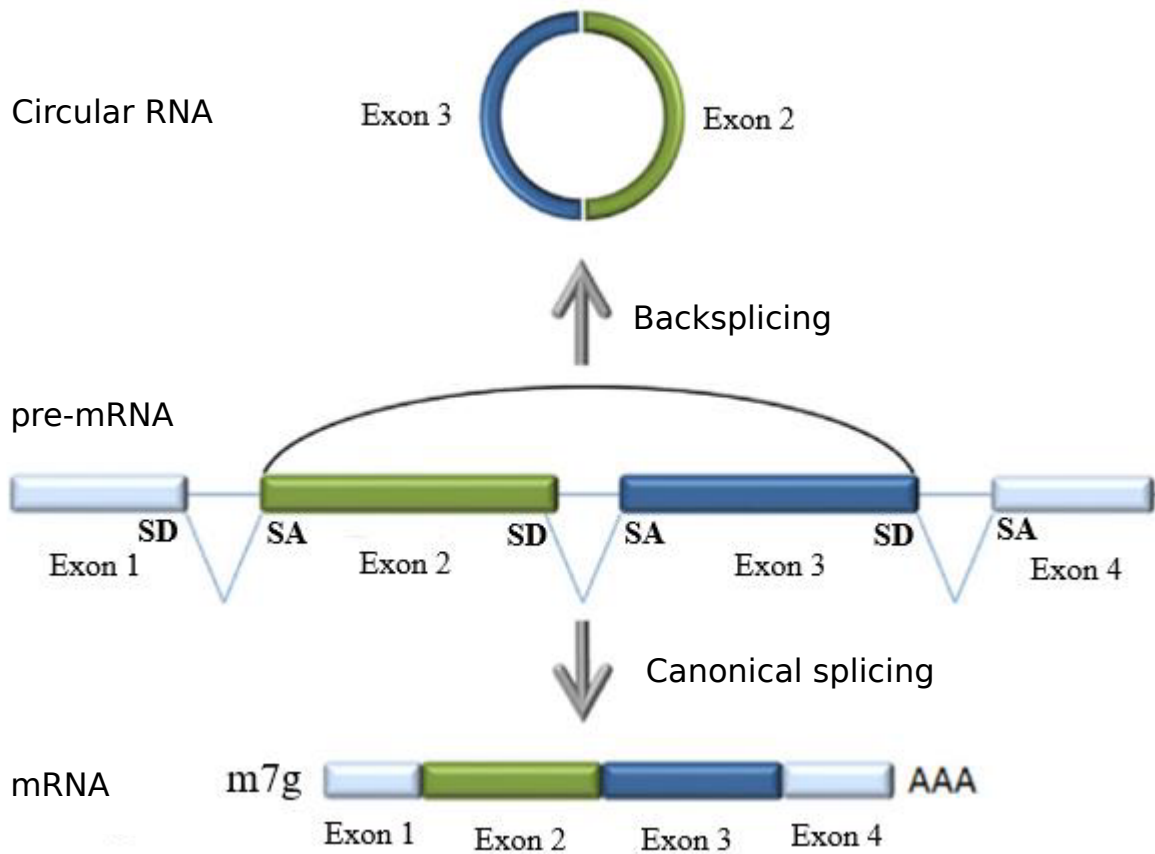


Figure 40. Schematic depiction of canonical (bottom) and backsplicing (top) of a pre-mRNA molecule. SD: splice donor. SA: splice acceptor. (From Yu and Kuo, 2019)

formed intronic RNA pairs to facilitate back-splicing as illustrated by the RBP Muscleblind (Mbl) which promotes production of circMbl from its own pre-mRNA in *D. melanogaster* (Ashwal-Fluss et al. 2014), or destabilize RNA base-pairing through their helicase activity as illustrated by the loss of DHX9 which increases the levels of circRNAs (Aktaş et al. 2017, 9). The proximity of the downstream donor to upstream acceptor sites can also arise from the presence of multiple RBPs which can interact with each other (D. Liang et Wilusz 2014). For example, multiple hnRNPs and SR proteins regulate back-splicing of the *laccase2* gene in *D. melanogaster* (Kramer et al. 2015). As a result of this process, circRNAs can either be constituted solely of RNA elements that would otherwise constitute exons, or include both exons and retained introns. (**Figure 41**)

The fact that these RNAs are looped implies they do not have any free extremities, and therefore are well protected from either 5'- or 3'-exonuclease degradation. They may indeed be very stable entities in a cell (Eneka et al. 2016). These highly stable transcripts contain almost exclusively exonic sequences and localize mainly to the cytoplasm. For some genes, the abundance of the circular RNA may exceed that of the associated linear RNA. The steady state abundance of circRNA is thus a balanced result of the poor efficiency of circRNA production, nuclear transport and low turnover. The expression of these molecules is therefore probably not very dynamic.

Since they share the same sequence as their related mRNAs, circRNAs likely have a common share of miRNA binding sites with them. Increased stability coupled with the usual absence of translating ribosomes on circRNAs makes them efficient to trap miRNAs and RBPs in a sponge-like manner. For this purpose, several miRNA binding sites are required to achieve a measurable effect in a given cell (Bosson, Zamudio, et Sharp 2014). The most striking example is the circular RNA CDR1as (also known as *CiRS-7*) which contains more than 70 conserved binding sites for miR-7 (Piwecka et al. 2017)(Piwecka et al. 2017). This circRNA is mainly expressed in the brain, but is also found in pancreatic islet cells where its overexpression can improve insulin expression by inhibiting the miR7-mediated insulin repression. This modification of miRNA availability can therefore affect their impact on gene expression (Hansen et al. 2013; Ashwal-Fluss et al. 2014). It is noticeable that binding sites for miRNAs in circRNAs are only partially complementary to miRNAs, allowing miRNA intracellular transport, but unproductive for Argonaute 2 targeting and further degradation. However circRNA can be degraded by different cytoplasmic endonucleases such as RNase L which is activated during viral infection (C.-X. Liu et al. 2019) or the ribonuclease complex RNase P/MRP known to be responsible for tRNA

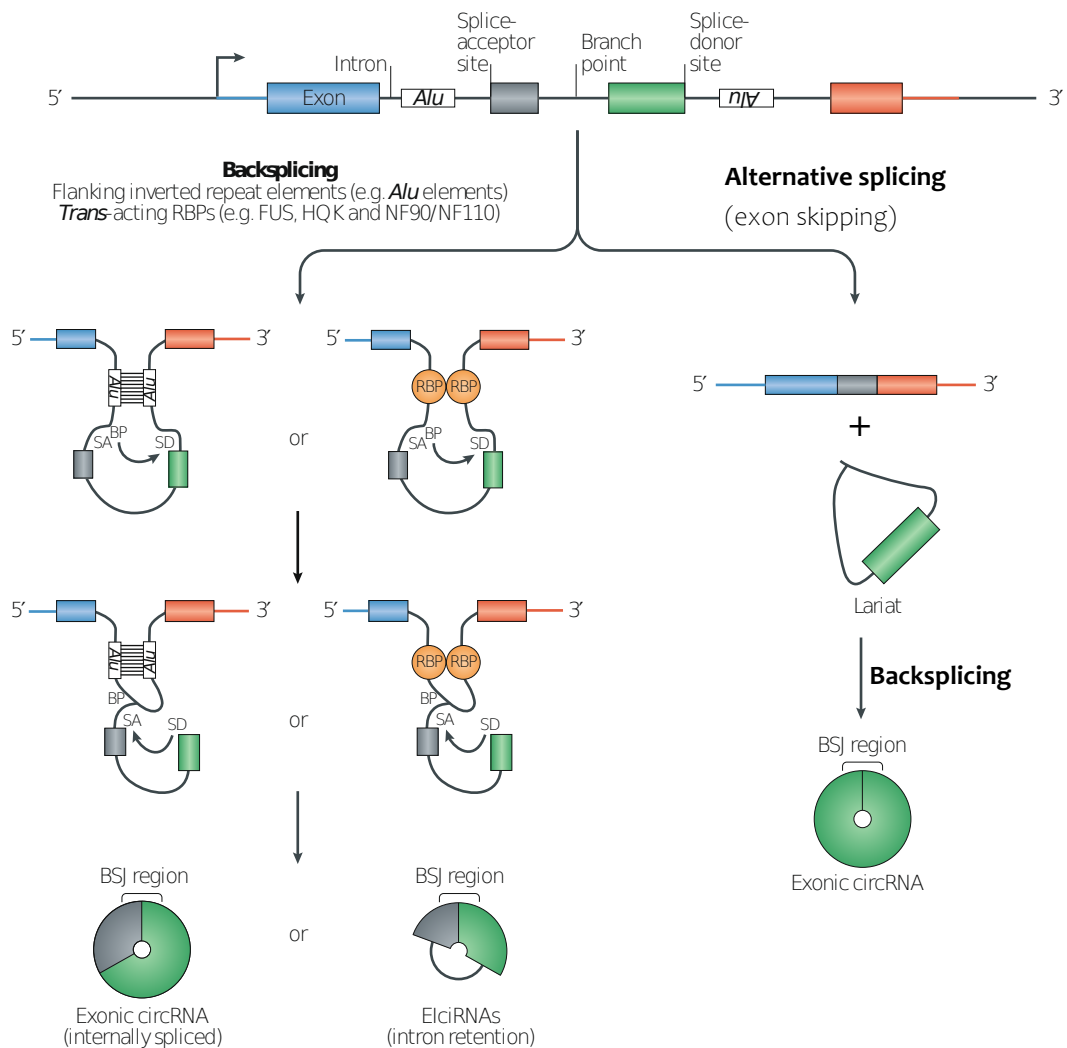


Figure 41. Circular RNA biogenesis. Donor and acceptor splice sites can be brought into close proximity by pairing of repeated sequences within their flanking introns and/or by RBP interactions. CircRNA can also be formed by the lariat generated by exon skipping during alternative splicing as it can bring the splice sites of the exon it contains together. RBP: RNA Binding Protein; BP: Branch Point; SD: Splice site Donor; SA: Splice site Acceptor; BSJ: Back Splice Junction; ElcircRNA: exon-intron circRNA (Adapted from Kristensen et al. 2019)

maturation and cleavage of ribosomal RNAs, long non-coding RNAs and mRNAs (Park et al. 2019). CircRNAs are also proposed to interact with RNA binding proteins in a scaffolding manner and further influence their cellular availability (Xiang Li et al. 2017; Du et al. 2016; 2017; Schneider et al. 2016). They may also encode peptides, provided they possess some internal ribosome entry sites (Lei et al. 2020). However circRNA-derived peptides are often truncated versions of the canonical proteins and lack essential functional domains. They may act as dominant-negative protein variants, decoys, or modulators of alternative protein complexes (Legnini et al. 2017).

CircRNAs have been implicated in numerous diseases including cancer, cardiovascular disease, autoimmune diseases and neurological disorders. (Zhou et al. 2020; Kristensen et al. 2019; L.-L. Chen et Yang 2015). An additional interest arises from the important stability of circular RNAs. They may indeed be sufficiently stable in blood to serve as circulating biomarkers that could be sampled without invasive procedures.

Recently, in 2019, a *TP63* circRNA was identified as potentially involved in lung squamous cell carcinoma (LUSC) development. This *TP63* circRNA (hsa-circ-0068515) is elevated in advanced stages of this disease. By binding to mir-873-3p, the *TP63* circRNA prevents this miRNA from decreasing FOXM1 levels, which is an inhibitor of cell cycle progression. This mechanism explains the increase of cellular proliferation and survival which follows the cytoplasmic increase of circ*TP63* in LUSC cells (Cheng et al. 2019). A number of other circRNAs have been identified in HNSCC, with a potential use as biomarkers (see **Table 5**) (Guo et al. 2019). Functions for circRNAs have also been described in head and neck cancer cells, controlling proliferation and the cell cycle, migration, apoptosis and response to chemotherapy either positively or negatively (Guo et al. 2019). Considering the apparent importance of TP63 in HNSCC biology, we sought to establish whether its circRNAs are expressed in HNSCC cell lines.

4.1. Identifying *TP63* circRNAs in HNSCC cell lines

From the *TP63* locus, Julia Salzman and colleagues identified three *TP63* circRNAs (Salzman et al. 2013). The first one originates from the TA promoter, hence I shall not detail it further. The second one, hsa-circ-0068515, contains exons 11 and 13 which surround the γ terminal exon. The third one, hsa-circ-0068516, contains exons 14 and 15 (**Figure 42.A**). I shall therefore refer to them as circ13-11 and circ15-14 for the sake of simplicity.

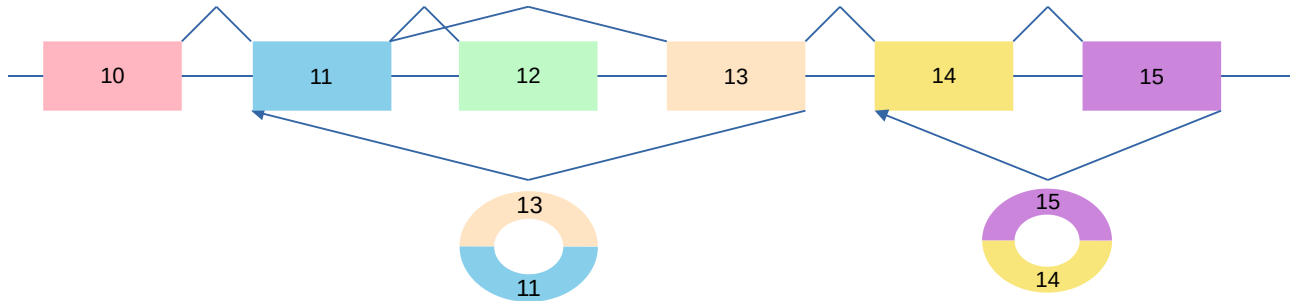
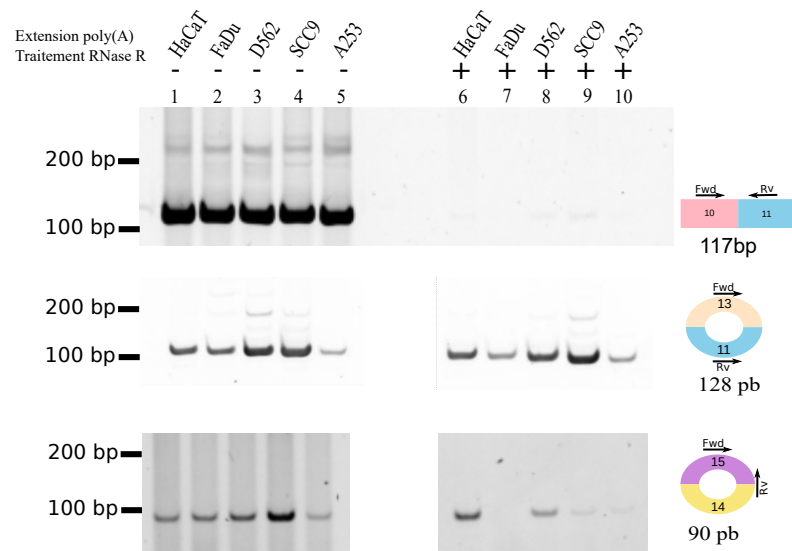
A**B**

Figure 42. A. Schematic depiction of circRNAs 13-11 and 15-14 production from the TP63 pre-mRNA **B.** Analysis of the abundance of TP63 mRNA (exons 10-11) and circs 13-11 and 15-14 by RT-PCR before and after poly(A) extension and RNase R digestion.

Table 5 The potential circRNA biomarkers in Head and Neck Cancers (From Guo et al. 2019)

CircRNAs	Chromosome	Gene symbol	Primary sites of cancer	Expression change	Relationships with the clinical features	Number of patients	Clinical samples	Clinical value
circPVT1	chr8	<i>PVT1</i>	HNSCC	Up	mut-p53, alcohol use	106	Tumor tissues	Poor overall survival (dependent on the TP53 mutations)
hsa_circ_0008309	chr2	<i>CUL3</i>	OSCC	Down	Pathological differentiation	45	Tumor tissues/ Non-tumor tissues	Diagnosis biomarker (AUC = 0.764)
hsa_circ_001242	chr10	<i>TRDMT1</i>	OSCC	Down	Tumor size, T stage	40	Tumor tissues/ Non-tumor tissues	Diagnosis biomarker (AUC = 0.784, Sensitivity = 0.725, Specificity = 0.775)
hsa_circ_0109291	chr19	<i>ZNF714</i>	OSCC	Up	TNM stage	51	Tumor tissues	Poor overall survival
hsa_circ_0001874	chr9	<i>BICD2</i>	OSCC	Up	TNM stage and tumor grade	178	Saliva from the OSCC patients and healthy controls	Early non-invasive diagnosis biomarker for OSCC in saliva (AUC = 0.863, Sensitivity = 0.744, Specificity = 0.902)
hsa_circ_0001971	chr7	<i>FAM126A</i>	OSCC	Up	TNM stage	178	Saliva from the OSCC patients and healthy controls	Early non-invasive diagnosis biomarker for OSCC in saliva (AUC = 0.845, Sensitivity = 0.756, Specificity = 0.878)
hsa_circ_0001874 + hsa_circ_0001971	-	-	OSCC	-	-	178	Saliva from the OSCC patients and healthy controls	Early non-invasive diagnosis biomarker for OSCC in saliva (AUC = 0.922, Sensitivity = 0.927, Specificity = 0.778)
hsa_circRNA_100855	-	-	LSCC	Up	T stage, lymph node metastasis, primary location, clinical stage	52	Tumor tissues/ Non-tumor tissues	Diagnosis and prognosis biomarker*
hsa_circRNA_104912	-	-	LSCC	Down	T stage, differentiation, lymph node metastasis, clinical stage	52	Tumor tissues/ Non-tumor tissues	Diagnosis and prognosis biomarker*
hsa_circ_0000284 (circHIPK3)	chr11	<i>HIPK3</i>	NPC	Up	Clinical stage, distant metastasis	63	Tumor tissues	Poor overall survival and distant metastasis-free survival rates
has_circ_0000285	chr11	<i>HIPK3</i>	NPC	Up	Tumor size, TNM stage, distant metastasis, tumor grade and lymph node metastasis	150	Serums and tumor tissues	Poor overall survival, poor radiosensitivity and serve as independent prognostic factors (HR = 3.03, $p = 0.02$)

Note: *HNSCC* head and neck squamous cell carcinoma, *OSCC* oral squamous cell carcinoma, *LSCC* laryngeal squamous cell carcinoma, *NPC* nasopharyngeal carcinoma, "-" means unannotated or not investigated in the paper, "*" means based on speculation but not validated clinically

Thanks to their circularity, circRNAs are resistant to the exonuclease activity of RNase R. Using this property, we depleted non-circRNA from total RNA purified from HNSCC cell lines and HaCaT cells by RNase R treatment. As suggested by Mei-Sheng Xiao and Wilusz, to maximise the degradation of structured linear RNA, an initial 3' polyadenylation reaction was performed to add an unstructured RNA handle to the 3' end of potentially structured RNAs (M.-S. Xiao et Wilusz 2019). Following this polyadenylation and RNaseR mediated degradation, we performed an RT-PCR analysis to detect linear and circular RNA molecules (**Figure 42.B**). cDNAs were produced with random hexamer priming. From cDNAs, exons 11 and 12 were amplified to detect *TP63* mRNA, and specific primers were designed to amplify circRNA. As shown in the top panel, RNase R digestion efficiently eliminates *TP63* linear RNA from the samples. The bottom two panels represent results for circ13-11 (middle panel) and for circ15-14 (bottom panel). For both circRNA, an amplified product at the expected size could be detected and appeared to be more resistant to RNaseR treatment than their linear counterpart. Sequencing of these PCR products positively identified them as being amplified from circRNA cDNA, since they possess a region specific to the back-spliced junctions (13→11 and 15→14) as indicated by the red arrows (**Figure 43**).

4.2. Does PTBP1 influence *TP63*'s back-splicing into circRNAs?

Of the many exons composing the *TP63* pre-mRNA, it is interesting that the two described circRNAs fall within regions subjected to alternative splicing. Since we previously identified PTBP1 as a regulator of *TP63* splicing, we sought to determine whether PTBP1 was also modulating the production of these circRNAs. By RT-qPCR, and using circ13-11 or circ15-14 back-splicing specific primers, we compared the abundance of circRNAs in PTBP1-depleted cells and control cells (**Figure 44 A, B**). For both circRNAs, depleting PTBP1 had no impact on their expression levels. Abundance of *TP63y* mRNA was increased in these same samples following PTBP1 depletion, serving as a positive control (**Figure 44 C**). One possible caveat is the fact that this analysis only detects presence or absence of the back-splicing junction, without knowing whether the RNA was in fact circular. Furthermore, this analysis was carried out on samples obtained after 48h of PTBP1 depletion. Since circRNAs are thought to be very stable, and their biogenesis infrequent, there may not have been enough time for an effect of PTBP1 depletion to be

A

```

réf. GCAACGCCCTCACTCCTACAACCATTCCCTGATGGCATGGGAGCCAACAGTGTGAGGGGCCGT 60
éch. -CAACGCCCTCACTCCTACAACCATTCCCTGATGGCATGGGAGCCAACAGTGTGAGGGGCCGT 59
      *****
réf. GAGACTTATGAAATGCTGTTGAAGATCAAAGAGTCCCTGGAATCATGCAGTACCTTCCT 120
éch. GAGACTTATGAAATGCTGT----- 86
      *****
réf.      CAGCACAC 128
éch.      ----- 86

```

Exon 13 TP63 Exon 11 TP63 ↓↑ Backsplicing junction

B

```

réf. GTTGGGCTGTTTCATCATGTCTGGACTATTTACGACCCAGGGGCTGACCACCATCTATCA 60
éch. -----ATGACATGAGCTGACCCTCTATCA 24
      *      ***      *****
réf.      GATTGAGCATTACTCCATGGATTTCCCATG 90
éch.      GATTGAGCATTACTCCATGGATTT----- 58
      *****

```

Exon 15 TP63 Exon 14 TP63 ↓↑ Backsplicing junction

Figure 43. Alignments of sanger sequences obtained from the PCR products of **A**. 13Fwd/11Rv or **B**. 15-14Fwd/14Rv reactions.

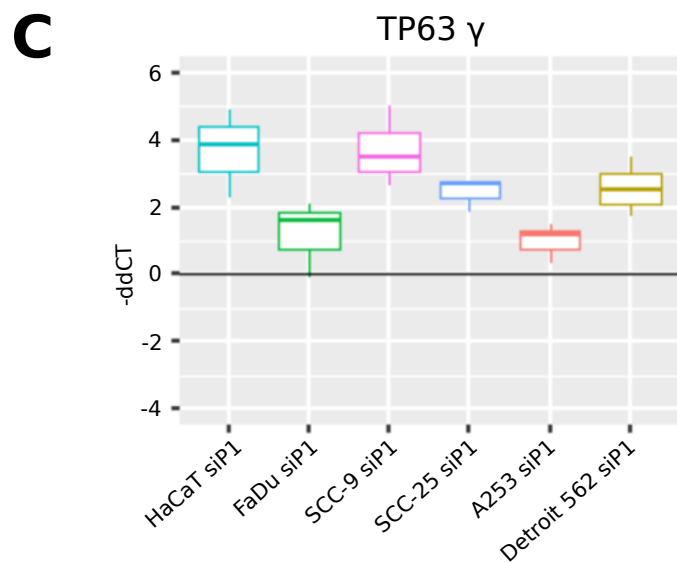
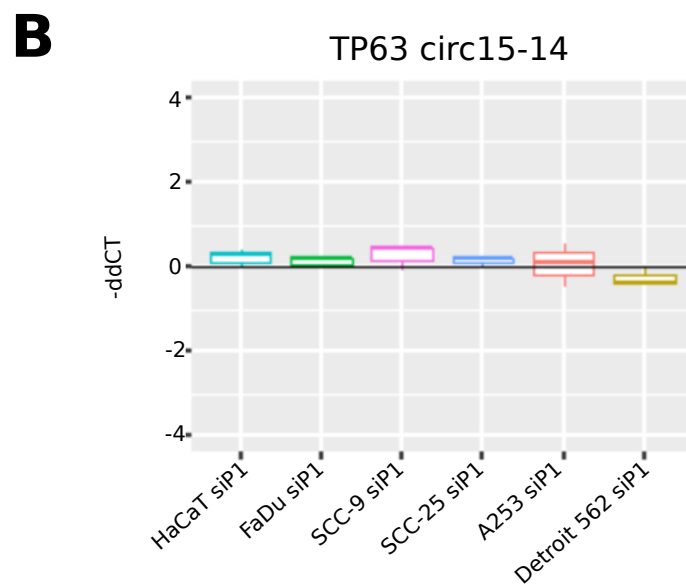
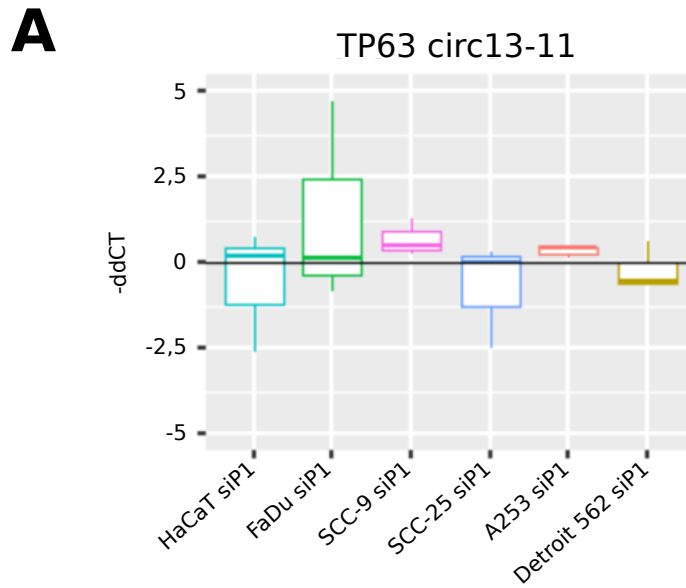


Figure 44. RT-qPCR measurement of circRNAs 13-11 (**A**) and 15-14 (**B**) expression in the indicated cell lines. TP63 γ expression was used as a PTBP1 target positive control (**C**). RNA was obtained from cells depleted in PTBP1 for 48h. Results were normalised to untreated cells and total TP63 was used as a reference gene following the ddCT method. siP1: siPTBP1.

visible on the pool of cellular *TP63* circRNAs. Analysis should therefore be carried out at further time points, and should also include other methods of detection such as northern blotting, which could allow us to conclude on the levels of circular RNA rather than on the sole presence of a back-splicing event.

4.3. Conclusion

TP63 circRNAs are indeed expressed in HNSCC cells, with preliminary evidence suggesting that their relative levels are just as variable as their mRNA counterparts. Interestingly, there does not seem to be a direct link between *TP63* abundance and *TP63* circRNA levels. A253 cells for example express high levels of *TP63*, yet they are almost the weakest in terms of circRNA expression. In view of this, it seems unlikely that circulating *TP63* circRNAs could directly reflect the exact expression of *TP63* within tumour cells. Since many normal epithelial tissues express *TP63*, there is likely a baseline level of circulating *TP63* circRNA in the body. However, it would be interesting to see whether variations of this baseline can be measured during tumour development, reflecting the overall number of *TP63* positive cells in the body. If this is the case, *TP63* circRNAs could become a useful tool for early detection of many epithelial derived tumours.

The apparent dissociation between *TP63* mRNA and *TP63* circRNA levels highlights the existence of some kind of regulation independent of *TP63*'s transcription rate. The circRNAs map to regions of the pre-mRNA which are subjected to alternative splicing. One possibility was therefore that the RNA binding protein PTBP1, which we have identified as a regulator of *TP63* alternative splicing, could also exert an effect on the biogenesis of *TP63* circRNAs. We tested this hypothesis by measuring the abundance of the respective back-splicing junctions or the *TP63* circRNAs with or without PTBP1 depletion and found no difference. From this experiment, it is however impossible to conclude to an absence of influence from PTBP1, as stated previously, and this will therefore be the object of further enquiries. It is yet unclear whether the production of *TP63* mRNA and *TP63* circRNA should be correlated or on the contrary inversely correlated as both biosynthetic pathways are exclusive.

The work of Cheng and colleagues has highlighted a function of *TP63* circRNA in a cancer context. It is however apparent that these circRNAs are not specific to cancer, we have notably detected them in HaCaT immortalised keratinocytes. It would be interesting

to see whether they are conserved across multiple *TP63*-expressing species which could reflect the evolutionary degree of importance of their regulatory pathways. Likewise, we have so far only identified these circRNAs in an epithelial context, and it would be interesting to assess their expression in non-epithelial *TP63*-positive cells such as muscle, bone or germline cells. In the context of HNSCC, this work will be carried on in the lab with overexpression experiments of circRNAs to try and identify phenotypic changes and their associated regulatory pathways, which will likely involve miRNAs.

GENERAL DISCUSSION

From a global point of view, the most striking finding of my Ph.D work is the identification of a roughly 400 million year old regulatory mechanism that mediates the inhibition of the *TP63 γ* isoform production. I have identified a conserved and functional regulatory interaction between the RNA binding protein PTBP1 and *TP63* alternative splicing. In tissues with high PTBP1 expression, splicing of *TP63* towards the longer C-terminal isoforms is favoured while in situations of lower PTBP1 expression the accumulation of an isoform deprived of most of the C-terminal domains is favoured. Obviously, the existence of this conserved and functional regulatory interaction between PTBP1 and *TP63* alternative splicing raises multiple fundamental questions that may not have any answer yet.

- *What is/are the function(s) of this shorter isoform that warranted its evolutionary conservation? And the corollary question: when did this alternatively spliced isoform emerge in the course of the TP53 gene family evolution?*

Answering these two questions will require tackling problems linked to developmental biology on one side, and to evolutionary biology on the other side. The increased accumulation of genomic and transcriptomic datasets from many different species across the tree of life should allow to identify the turning point when *TP63* gamma emerged. Was it before the divergence of *TP63* and *TP73* functions or was it later on, once the family was already composed of the three members (*TP73/TP63/TP53*)? The latter hypothesis would indicate that p63 γ specific functions are likely linked to the expression of the other family members. *TP73*'s expression pattern has been shown to overlap with *TP63*'s in some situations (Marshall et al. 2021). p73 participates in developmental processes that also require p63 activity such as the development of mucociliary epithelium. In this context, p63 γ could be implicated in the fine tuning of certain developmental steps, outside of which its expression would be of limited use to cells. This would be in line with its overall absence in adult tissues, skeletal muscles aside. It would also explain the lack of any striking modifications in cellular phenotype following its overexpression as shown in the second results chapter. Furthermore, p63 γ expression in striated skeletal muscle cells has been shown to be cytoplasmic, which contrasts with the general assumption that p63 always acts as a transcription factor. In the few instances

where it is expressed, p63 γ is present as a TA isoform. This raises two possibilities: γ 's use could be restricted to the TA isoform with no real requirement for the Δ N isoform, or γ splicing could be more efficient in the context of transcription from the TA promoter.

Tackling developmental functions of the γ isoform will require specific alteration of its expression in a complex organism. *TP63* isoform specific knockouts mediated by CRISPR CAS could probably allow us to perform such gene editing, and promote only *TP63 α* expression. *Xenopus* could be a model of choice for this work as embryos allow rapid access to developmental stages pertinent for *TP63* biology and efficient F0 genome editing. This would be all the more interesting since *TP63* isoform specific knockouts and re-expressions have already been performed in mice, which did not highlight any strong *TP63 γ* related phenotypes. Direct access to the developing embryos could facilitate the observation and characterisation of potentially subtle effects, which may have been missed in a mouse model if embryo analysis was performed at the wrong stage. Analysis of *Xenopus* embryos could allow us to determine in which tissue or under which circumstances the gamma isoforms are required. If p63 γ 's function is indeed limited to a small number of cells, single cell RNAseq analysis could allow us to identify the cells of interest within an affected tissue. While SC-RNAseq is usually not indicated for analysis of alternatively spliced isoforms, alternative terminal exon usage can be discriminated by the classical sequencing techniques used in single cell analysis.

- *Does the gamma isoform have a regulatory role on TP63 α ?*

In the course of the experiments described in this thesis, we showed that specific downregulation of *TP63 γ* led to a decrease in *TP63 α* . While ruling out a potential artefact would require additional experiments, the fact that two different siRNAs targeting *TP63 γ* were capable of directing decreased *TP63 α* expression points to a specific effect event though its mechanism is unclear. In particular it is obscure whether this mechanism is mediated by a drop in p63 γ protein expression or whether it's mediated directly by the siRNA used. Previous work showed that HeLa cells overexpressing *TP63 γ* started to express *TP63 α* (Antonini et al. 2006). We should reiterate these experiments, forcing *TP63 γ* expression in HeLa cells, with concomitant knockdown of *TP63 γ* by siRNA to ascertain that p63 γ is indeed involved in a self amplifying loop for *TP63 α* activation.

- *Does p63's c-terminal region affect its subcellular localisation?*

In human adult tissues, the sole robust expression of *TP63 γ* is observed in muscle tissues. Interestingly, rhabdomyosarcomas that are cancer derived from muscle cells are characterized by accumulation of p63 in a somehow unusual localisation as it appears to be cytoplasmic (Martin et al. 2011). Abnormal cytoplasmic localisation of TP63 has been associated with poor outcome in prostate cancer patient (Dhillon et al. 2009) and in lung adenocarcinoma (Narahashi et al. 2006). However it is unclear the nucleo-cytoplasmic relocalisation of p63 is mediated. Actually, it was unclear until recently how proper nuclear localisation of p63 is achieved (Hazawa et al. 2018). In rhabdomyosarcomas, is the cytoplasmic localisation linked to the nature of the C-terminal region of p63 isoforms, or to altered regulation of the import pathway under the control of NUP62 and Rho Kinase (Hazawa et al. 2018)? The specific localisation of p63 at the Z-striations in muscle cells suggests it is not merely present in the cytoplasm through lack of nuclear import; it would rather seem that p63 is purposefully addressed to this location to ensure a yet unknown function. It would be interesting to express different p63 in a muscle cell line, and follow their subcellular localisation following differentiation.

- *Considering our demonstration of TP63 γ repression by PTBP1 in epithelial cells, what consequence would PTBP1 expression have on TP63 isoforms produced in muscle cells?*

We have mainly addressed the control of PTBP1 on *TP63* alternative splicing using loss of function methods. The model we propose of repression of the *TP63 γ* isoform splicing by PTBP1 would gain to be challenged using gain of function experiments. It could be particularly interesting to symmetrically address the question using gain of function of PTBP1 in a muscular context to determine if accumulation of *TP63* alpha consequentially occurs. A muscular cell model would also allow for the investigation of the participation of other RNA BPs on *TP63* splicing, most notably potential activators of *TP63 γ* splicing.

- *Does regulation of TP63 alternative splicing have potential for diagnostic or therapeutic use?*

While overexpression of *TP63* is associated to specific types of cancer, most notably in squamous carcinomas (lung, head and neck, cervical) and oesophageal adenocarcinomas, disease progression is usually correlated to loss of *TP63* expression. We showed that in HNSCC patients, a high inclusion of *TP63 γ* is associated with a shorter overall survival of the patients. While this does not demonstrate a causal link between the

two phenomenon, this does however suggest that analysis of *TP63* expression in tumours samples could allow refinement of tumour description and grading. However, no antibodies are so far available to address this question. The alternative to immunostaining would be to analyse the expression of *TP63* isoforms at the RNA level in FFPE samples using RT-qPCR. Of course, it has to be noted that due to the correlative nature of the relationship between *TP63 γ* expression and patient survival, *TP63 γ* could have no causal affect at all. Instead, it could be the result of an overall modified splicing landscape in cancer cells. The expression of the correct set of RNA BPs is an important element in cellular differentiation. In cancer, disease progression is usually associated with a de-differentiation of cells, or trans-differentiation into aberrant cell types for the tissue of origin. In this context, measuring *TP63 γ* levels could still be used as an interesting marker of aberrant splicing in epithelial cancer cells.

- *TP63 alternative splicing: an entry point for disease producing mutations?*

The regulatory mechanism we have identified likely controls some *TP63* functions. Apart from carcinogenesis, mutations in *TP63* are causal to many different developmental syndromes affecting ectodermal derivatives. Among the repertoire of different syndromes, the location of the mutation relative to the functional domains of the encoded protein defines the spectrum of individual defects. One can envisage that mutations located in intronic regulatory domains affecting PTBP1 binding to *TP63* could promote expression of the γ isoform. This could define *TP63* dependent pathological consequences which should resemble those stemming from mutations producing truncated *TP63* proteins. In a broader sense, mutations which increase the strength of the *TP63 γ* exon's splice sites in cis or in trans could explain cases of patients showing a p63-attributed disease without having any mutations in regions corresponding to functional domains.

- *Is TP63 functionally important besides its protein products?*

There are two striking differences between *TP63 α* and *TP63 γ* . The first, and most obvious, is the lack of c-terminal regulatory domains in p63 γ . This has long stood as an argument for a dominant-negative like role of γ on α . The experimental proof of this has yet to emerge. Furthermore, in an adult body, *TP63 γ* is expressed in muscle cells where other isoforms are absent. This obviously circumvents any possible dominant-negative

functions. The second difference lies in the difference in 3'UTR between *TP63 α* and γ . 3' UTRs are notoriously involved in post-transcriptional control of RNAs by binding to complementary sequences or by recruiting protein factors. Their wide ranging regulatory action comes from influencing processes such as mRNA localisation, stability and translation. Part of their function is mediated through recruitment of miRNAs. Besides 3'UTRs, we have also shown that circRNAs derived from the *TP63* pre-mRNA are expressed in HNSCC cell lines. These circRNAs share their sequence with some *TP63* exons. In this circularised form, regulatory sequences which they may contain are protected from degradation. The existence of this stabilised forms of *TP63 RNA* could indicate that they possess regulatory functions which are totally independent from the protein.

In conclusion, we have established that the production of the *TP63 γ* isoform has been tightly regulated for hundreds of millions of years by the RNA binding PTBP1. The strong conservation of its regulation contrasts with its overall extremely low expression. This suggests that whatever the function of *TP63 γ* , it is generally dispensable, but must be fundamentally important in one or several contexts to warrant its continued existence. Despite previous descriptions of its involvement in the EMT, we have been incapable of reproducing such effects. In fact, we have been incapable overall of identifying a *TP63 γ* specific functions in normal or cancerous epithelial cells. Coming out of this work, many questions therefore remained unanswered. Where and when is $\Delta Np63\gamma$ expressed? To what end? How is specific *TP63 γ* expression driven in muscles; what are its activators? These questions will require the development of both novel cellular and animal based models, and would greatly benefit from the successful development of isoform specific antibodies in the future.

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RÉSUMÉ FRANÇAIS

I. Contexte bibliographique de la thèse

Au sens commun du terme, une espèce est un ensemble d'individus ayant pour caractéristique de pouvoir se reproduire entre eux tout en donnant une descendance elle-même fertile. Ces propriétés requièrent une certaine forme de stabilité ou d'intégrité du patrimoine génétique. Le contrôle de l'intégrité de ce patrimoine génétique revêt deux aspects. Le premier est la capacité d'un individu donné à résister aux dommages subits au quotidien par son ADN, dommages qui, s'ils n'étaient pas réparés, pourraient être à l'origine de cancers. Le second, est la capacité, en tant qu'espèce, à conserver un patrimoine génétique qui puisse être transmis de génération en génération. Le premier cas nécessite donc des éléments de contrôle des dommages à l'ADN au niveau somatique, le second au niveau germlinal. Cette pression pour un maintien de l'intégrité génétique est certainement l'un des moteurs qui a favorisé l'apparition précoce, au cours de l'évolution, de la famille de facteurs de transcription p53 dont on retrouve un gène ancestral tant chez les choanoflagellés unicellulaires que chez les métazoaires comme les cnidaires. Aux côtés de *TP53*, éponyme de la famille, on trouve deux autres gènes : *TP63* et *TP73*. Ces trois gènes sont apparus par des événements de duplication, et leur phylogénie est détaillée en **Figure 1**. Au niveau protéique, les membres de la famille p53 partagent une organisation en domaines fonctionnels tout à fait comparable avec un domaine N-terminal de transactivation, un domaine central de liaison à l'ADN, un domaine impliqué dans leur oligomérisation et plusieurs domaines C-terminaux impliqués dans leurs régulations. En particulier, le domaine de liaison à l'ADN est très conservé entre les trois membres. Leurs spécificités fonctionnelles ont été largement appréciées grâce à des expériences de délétion de gènes chez la souris, et sont mises en relation avec des observations faites chez l'homme lors de la présence de mutations dans les différents membres de la famille. Les souris *i* n'exprimant plus de gène fonctionnel *TP53* n'ont pas de problèmes de développement mais accusent une nette hausse d'apparition précoce de tumeurs de multiples origines comparé à leurs congénères sauvages. Chez l'humain, la mutation de *TP53* est la cause du syndrome de Li-Fraumeni, une maladie autosomique récessive rare qui se caractérise par une augmentation importante du risque de cancer. D'une façon plus large, 50 % de tous les cas de cancers portent des mutations sur le gène *TP53*. La

délétion de *TP63* ou *TP73* chez la souris ne s'accompagnent pas d'une hausse de survenue de tumeurs, mais plutôt de graves problèmes développementaux. Dans le cas de *TP63*, ces défauts de développement touchent divers dérivés ectodermiques dont la peau, les membres, et la face, tandis que les souris qui ne peuvent plus exprimer de *TP73* sont affectées au niveau du système nerveux central et du système immunitaire. Chez l'homme, des mutations de *TP63* sont responsables d'un panel de syndromes développementaux partageant des dysplasies ectodermiques. De façon globale, p53 est donc responsable du contrôle de l'intégrité génétique somatique, tandis que p63 et p73 sont responsables de programmes développementaux au niveau ectodermique et au niveau du système nerveux central respectivement.

II. Situation du sujet de la thèse

La structure et la régulation des gènes de la famille *TP53* sont complexes. Au moins deux promoteurs sont décrits pour chaque gène, auxquels s'ajoutent de nombreux événements d'épissage alternatif et parfois également de traduction alternative. Au total, ces mécanismes génèrent 12 isoformes de p53, environ 30 isoformes de p73, et au moins 8 isoformes de p63 (**Figure 3**).

Au cours de cette thèse, je me suis intéressé à la régulation et à la fonction des isoformes de *TP63*. Les isoformes qui possèdent le domaine de transactivation N-terminal sont produites à partir du premier promoteur (P1) et sont appelées TAp63. Celles qui sont dépourvues de ce domaine de transactivation, produites à partir du second promoteur (P2), sont appelées Δ Np63. Les protéines de ces deux catégories peuvent exister en quatre isoformes C-terminales différentes : une isoforme pleine longueur, α , et trois isoformes présentant des extrémité C-terminales partielles : β , γ et δ . Ces isoformes C-terminales sont issues d'évènements d'épissage alternatif.

L'épissage alternatif s'inscrit dans le processus de maturation des ARN pré-messagers, au même titre que l'adjonction d'une coiffe de 7-méthylguanosine en 5' et de leur clivage et polyadénylation à leur extrémité 3'. L'épissage consiste à rabouter séquentiellement des portions de l'ARN pré-messager entre elles, qui constitueront les exons, et à en éliminer d'autres régions de l'ARN pré-messager qui constituent les introns. Cette opération nécessite la reconnaissance de quatre séquences par les acteurs moléculaires de l'épissage. Ce sont le site 5' d'épissage, défini par la séquence AG/GURAGU, le site 3' d'épissage YAG/xx, une séquence riche en pyrimidines située 10 à

20 nucléotides en amont du site 3' d'épissage, et le point de branchement constitué par une adénine au sein de la séquence YNYURAY. Grâce à ces éléments, une structure complexe et hautement dynamique constituée de petits ARN et de protéines appelée le spliceosome va pouvoir reconnaître les bornes entre les exons et les introns, et catalyser l'assemblage des exons entre eux et l'excision des introns. L'épissage est dit alternatif lorsque la réaction d'épissage va préférentiellement se faire entre certains exons plutôt que d'autres. Certains exons seront alors exclus de l'ARNm mature ce qui permet de générer une diversité d'ARN messagers et donc de protéines, tel qu'il est observé pour p63 (**Figure 4**). L'épissage alternatif est possible grâce aux différences d'efficacité existantes entre les sites d'épissage de différents exons. En effet, les séquences peuvent être au moins partiellement dégénérées et peuvent ainsi promouvoir l'association avec les composants du spliceosome de façon plus ou moins efficace. Ainsi, au sein d'un ARN pré-messager on peut distinguer les exons dont la force des sites d'épissage est suffisante pour assurer leur épissage, ces exons sont dits constitutifs, et d'autres dont la force est plus faible et qui peuvent donc être en compétition les uns avec les autres au sein d'événements d'épissage dits alternatifs. La force des différents sites d'épissage, en tant que capacité à être reconnus par les acteurs du spliceosome peut être influencée par des séquences cis annexes, localisées dans les introns ou les exons. Ces séquences cis régulatrices servent à recruter des activateurs ou des répresseurs de l'épissage qui agiront localement sur l'utilisation de certains exons.

D'une manière similaire à l'épissage, le clivage et la polyadénylation d'un ARN pré-messager nécessitent des éléments de séquence bien définis. Ce sont le site de clivage, CA, le signal de polyadénylation, généralement l'hexanucléotide AAUAAA, situé 10 à 30 nucléotides en amont du site de clivage, ainsi qu'une région riche en G/U et située environ 30 nucléotides en aval du site de clivage. Un ARN pré-messager peut posséder plusieurs sites de clivage et polyadénylation qui pourront être utilisés différemment en fonction des conditions ou des types cellulaires, on parle alors également de clivage et polyadénylation alternatif.

En termes d'expression, les isoformes TAp63 sont presque exclusives aux testicules, aux muscles striés squelettiques et aux cellules germinales femelles. Les isoformes Δ Np63 sont exprimées principalement dans les tissus épithéliaux. Bien qu'elles soient dépourvues du domaine de transactivation N-terminale, les isoformes Δ Np63 possèdent tout de même un autre domaine de transactivation (Figure 2). Ainsi, ces deux

groupes d'isoformes sont tous les deux capables de réguler la transcription. Les cibles de ces 2 types d'isoformes doivent être cependant suffisamment différentes pour expliquer leurs spécificités de fonctions. Ainsi, les isoformes TA sont surtout décrites pour leur rôle dans la surveillance de l'intégrité génomique des cellules germinales, tandis que les isoformes ΔN sont surtout connues pour leur capacité à promouvoir le caractère souche de cellules de la couche basale d'épithéliums stratifiés. Du côté C-terminal, l'isoforme pleine longueur α est de loin la plus représentée, suivie loin derrière par β , puis par γ . L'isoforme δ n'est quant à elle quasiment jamais décrite. L'immense majorité des effets démontrés pour p63 sont attribuables à l'isoforme α . Néanmoins, quelques fonctions ont été également attribuées à l'isoforme γ , notamment lors de la formation des myotubes dans les muscles striés squelettiques ainsi que lors de la différenciation des chondrocytes.

III. Résumé des résultats obtenus lors de la thèse

Dans le contexte du cancer, la majorité des sources s'accordent à attribuer à $\Delta Np63\alpha$ un rôle anti-apoptotique, anti-migratoire, et pro-prolifératif. Ses fonctions seraient ainsi particulièrement utiles au développement initial d'une tumeur primaire. *TP63* est fréquemment amplifié et/ou surexprimé dans les carcinomes épidermoïdes, mais dans les cas avancés son expression est généralement perdue. De façon intéressante, l'isoforme γ pourrait avoir des fonctions en opposition avec α puisqu'un rôle pro-transition épithélio-mésenchymateuse lui a été décrit dans plusieurs lignées cellulaires. Par analyse de données de RNAseq de la cohorte HNSC du TCGA, nous avons mis en évidence dans les données tumorales une corrélation négative entre la proportion d'isoforme γ exprimée par rapport aux autres isoformes et les chances de survie des patients porteurs de ces tumeurs.

En termes de régulation de la production et de l'abondance des différentes isoformes, les connaissances actuelles sont plutôt limitées. Au niveau de l'ARN, la 3'UTR différente entre γ d'une part et α et β d'autre part permet leur régulation différentielle. Une protéine de liaison aux ARN, Rbm38, est notamment connue pour stabiliser l'ARNm *TP63* γ et déstabiliser l'ARNm α et β . Plusieurs miRNA ont également été décrits dans la régulation de *TP63*, notamment mir203 qui cible la 3'UTR partagée par α et β . Au niveau protéique, plusieurs E3 ligases dont les sites de reconnaissance se trouvent dans le domaine SAM, absent de l'isoforme γ , ont également été décrites. Ce sont notamment Nedd4, WWP1 ou Fbw7. Aucune information n'existe quant à la régulation des

événements d'épissage alternatif conduisant à la genèse des différentes isoformes C-terminales de *TP63*.

Afin d'identifier des régulateurs de l'épissage alternatif de *TP63*, et à partir des données d'expression du consortium GTEX, nous avons quantifié l'utilisation des 21 jonctions exon-exon possibles de *TP63* à travers les différents tissus où il est exprimé. La jonction conduisant à la synthèse de l'isoforme γ est notamment en compétition avec celle conduisant aux autres isoformes C-terminales. Cette jonction est majoritaire dans le muscle, alors que dans les autres tissus c'est à l'inverse celle conduisant aux isoformes α et β qui l'est. Nous avons alors émis l'hypothèse que l'analyse des corrélations entre les protéines de liaison aux ARN exprimées et l'utilisation de l'une ou de l'autre de ces jonctions nous permettrait d'identifier certains de ses régulateurs potentiels. Nous avons sélectionné les 10 protéines de liaison aux ARNs dont l'expression était la plus corrélée à l'utilisation de la jonction γ . Nous avons alors analysé l'expression de ces dix protéines dans les tumeurs classées comme ayant beaucoup ou peu de *TP63* γ . A l'issue de ces deux étapes, la PTBP1 est apparue comme un bon candidat en tant que répresseur de l'inclusion de l'exon terminal γ .

Afin de vérifier si la PTBP1 réprime effectivement l'utilisation de l'exon terminal γ , nous avons bloqué son expression dans des lignées cellulaires issues de carcinomes épidermoïdes de la tête et du cou (HNSCC). Pour chacune des lignées testées, la déplétion de PTBP1 pendant 48 h s'est accompagnée d'une augmentation significative de l'expression de *TP63* γ , mesurée par RT-qPCR. Nous avons également montré par des expériences de retard sur gel utilisant de la PTBP1 recombinante et des fragments d'ARN *TP63* que celle-ci peut se fixer à des régions en amont et en aval de l'exon γ *in vitro*. Ces régions correspondent à des séquences génomique présentant une forte conservation chez les vertébrés. La détection par RT-qPCR d'un enrichissement en ARN pré-messager de *TP63* suite à l'immunoprécipitation de la PTBP1 nous a prouvé que cette interaction directe pouvait également avoir lieu dans les cellules. Afin de vérifier si les régions introniques flanquant immédiatement l'exon gamma étaient suffisantes à sa régulation par la PTBP1, nous avons construit un minigène constitué de la séquence génomique comprenant l'exon γ dans le contexte robuste d'épissage de la beta-globine. Lorsqu'il est exprimé dans la lignée de kératinocytes HaCaT, la proportion d'inclusion de l'exon γ dans les transcrits issus de ce minigène augmente lorsque l'on déplete la PTBP1. Ceci prouve

que la région génomique comprise dans ce minigène est suffisante pour permettre la régulation de *TP63* γ par la PTBP1.

La structure du gène *TP63* est bien conservée entre le xénope et l'homme, avec la présence d'un exon terminal γ chez les deux espèces. L'analyse des séquences génomiques suggère une conservation des régions comprenant les sites de liaison de la PTBP1 que nous avons identifié au travers de nombreuses espèces, dont le Xénope. Par l'utilisation de morpholinos antisens, nous avons déplété la PTBP1 dans des embryons de Xénope avant d'analyser leur transcriptome par RNAseq. Il s'avère qu'à la suite de la déplétion de la PTBP1 dans ces embryons, le niveau de *TP63* γ augmente. Le mécanisme de répression de l'inclusion de l'exon terminal γ par la PTBP1 est donc un mécanisme conservé au cours de quasiment 400 millions d'années d'évolution. Une conservation aussi forte suggère un rôle biologique important de la production de l'isoforme γ de *TP63*. Nous avons donc voulu en savoir plus sur la fonction cette isoforme .

Dans la littérature, plusieurs sources s'accordent à attribuer à p63 γ un rôle de promoteur de la transition épithélio-mésenchymateuse (EMT). Cette transition correspond à un changement phénotypique qui fait passer d'une cellule épithéliale à une cellule mésenchymateuse. Cette EMT résulte de modifications transcriptionnelles et post-transcriptionnelles qui, ensemble, modifient la polarité de la cellule, la force des adhésions intercellulaires, le cytosquelette, et plus globalement les capacités migratrices et invasives des cellules. C'est un processus physiologique clé lors du développement embryonnaire mais également lors de la réparation tissulaire. Dans les quinze dernières années, ce processus a également été très étudié pour sa participation à la dissémination métastatique des tumeurs primaires. L'isoforme γ de *TP63* étant uniquement exprimée de façon significative dans les muscles, et réprimée dans les tissus épithéliaux par la PTBP1, il nous a paru pertinent d'évaluer le rôle de l'isoforme *TP63* γ et de sa régulation par la PTBP1 dans le contexte de l'EMT en utilisant le modèle des cellules HaCaT (des kératinocytes normaux immortalisés) et des cellules SCC-9 (carcinome lingual). Nous avons pour cela comparé la morphologie cellulaire et l'expression de marqueurs épithéliaux et mésenchymateux au niveau ARN et protéique entre des cellules exprimant plus de *TP63* γ (soit suite à l'inactivation de PTBP1, soit suite à sa surexpression par transfection plasmidique) et des cellules induites en EMT par un traitement au TGF- β . Au cours de ces expériences, nous n'avons identifié aucun effet de l'abondance de *TP63* γ sur le phénotype épithélial des cellules.

Afin de déterminer si une modification de la proportion de *TP63* γ dans les cellules HNSCC pouvait avoir des conséquences fonctionnelles sur la cellule, nous nous sommes focalisés sur des gènes cibles précédemment décrits pour p63. Par inactivation et surexpression isoforme-spécifique de *TP63*, nous avons cherché à identifier lesquels de ces gènes pouvaient être contrôlés par p63 α , p63 γ ou les deux isoformes. Contre toute attente, nous n'avons observé que des effets très modérés de la modification de l'abondance des isoformes de p63 sur les cibles que nous avons choisies. Par ailleurs, la déplétion de *TP63* γ s'accompagnant également d'une baisse de *TP63* α , il nous a été impossible d'identifier avec confiance des gènes qui seraient la cible spécifique de l'une ou l'autre de ces isoformes.

Compte tenu de l'association que nous avons mise en évidence entre le niveau de *TP63* γ et le pronostic des patients atteints de HNSCC, il serait important de tester la pertinence de la détection de p63 γ dans les échantillons de patients dans un but diagnostique. Ce type d'approche repose généralement sur le marquage immunohistologique de coupe de tissu de patient. Ceci requiert bien entendu un anticorps capable de reconnaître spécifiquement la cible d'intérêt, en l'occurrence p63 γ . Bien qu'il existe une courte séquence peptidique spécifique de l'isoforme γ , aucun anticorps commercialisé ne lui est aujourd'hui spécifique. Le choix se limite à des anticorps capables de reconnaître toutes les isoformes C-terminales, ou bien uniquement la forme α . En partenariat avec l'établissement français du sang, nous avons donc cherché à développer de nouveaux anticorps monoclonaux ciblant p63 et ce de façon isoforme-spécifique. Après immunisation de souris avec des peptides spécifiques à p63 α , p63 γ ou communs à toutes les isoformes (pan p63), nous avons sélectionné celles dont le sérum reconnaissait à la fois les peptides d'intérêt en ELISA, et les isoformes correspondantes surexprimées en cellules HeLa par western blot. A l'issue de cette phase du projet, nous avons généré des hybridomes à partir des souris les plus prometteuses en termes de spécificité et de sensibilité. L'analyse des surnageants d'hybridome, par ELISA et par WB comme précédemment, a abouti au choix d'un clone pan p63, le KO1393, et d'un clone spécifique à p63 γ , le KO85. Ces clones ont effectivement montré une bonne sensibilité et une bonne spécificité vis à vis de protéine p63 surexprimée en cellules HeLa, que ce soit par western blot ou par immunofluorescence. En revanche, lorsque nous avons testé ces anticorps pour la reconnaissance d'isoformes de p63 moins fortement surexprimées, ou pour la reconnaissance de p63 endogène nous avons été confrontés conjointement à un manque

de sensibilité et de spécificité de ces anticorps. Par western blot le signal non spécifique dépasse notablement le niveau du signal spécifique. Pour aller plus loin, une perspective pourrait être de faire évoluer ces anticorps par mutagenèse, en sélectionnant les clones ayant la meilleure sensibilité et spécificité vis à vis de p63. A terme, et au-delà de leur potentielle utilisation clinique, de bons anticorps spécifiques des isoformes de p63 seront sans doute la clé vers l'élucidation de leurs fonctions protéiques propres.

Outre la production de plusieurs ARNm permettant la synthèse de plusieurs isoformes protéiques, le locus *TP63* est également à même de produire trois ARN circulaires. Les ARN circulaires sont produits à partir d'un épissage non canonique appelé épissage rétrograde au cours duquel un site 3' donneur d'épissage réagit avec un site accepteur d'épissage localisé en amont sur l'ARN prémessager. Du fait de leur circularité, ces ARN ne sont pas sensibles aux exonucléases et sont très stables. Ils peuvent donc potentiellement être abondants en dépit de la faible efficacité de l'épissage rétrograde comparée à l'épissage classique. Du fait de leur abondance et de leur stabilité, les ARN circulaires peuvent efficacement séquestrer des miRNA ou des protéines de liaison aux ARNs. De façon intéressante, l'absence de queue polyA rend les ARN circulaires insensibles à une dégradation polyA-nucléasique telle qu'il pourrait être observé suite à la fixation d'un miARN sur un ARN linéaire. La fixation des miARN sur les ARN circulaires ne déclenchent donc a priori pas d'instabilité. Dans les rares cas où ils possèdent des sites internes d'entrée du ribosome, les ARN circulaires peuvent être traduits en de petits peptides. Par ces fonctions, les ARN circulaires ont été impliqués dans diverses pathologies dont le cancer, les maladies cardiovasculaires, autoimmunes et neurologiques. Ils pourraient également en être de prometteurs biomarqueurs sanguins, offrant l'avantage d'une bonne stabilité et associée avec la facilité du prélèvement sanguin. L'ARN circulaire hsa-circ-0068515 de *TP63*, qui comprend les exons encadrant l'exon terminal γ , a été identifié dans le développement de carcinomes épidermoïdes pulmonaires. De nombreux ARN circulaires ont été identifiés dans les HNSCC avec un rôle soit fonctionnel, soit une pertinence en tant que biomarqueur. Compte tenu de l'importance de *TP63* dans les HNSCC, nous avons donc voulu déterminer si les ARN circulaires qui en découlent sont exprimés dans ce type de cellules. Aussi, puisque nous avons identifié PTBP1 comme régulateur de l'épissage de *TP63*, nous avons testé son effet sur la genèse de ces ARN circulaires. Suite à la dégradation par Rnase R des ARN linéaires dans des échantillons issus de nos cinq lignées de HNSCC, ainsi que de cellules

HaCaT, nous avons mesuré l'expression de deux ARN circulaires *TP63* par RT-qPCR. Ces ARN circulaires sont exprimés, à des niveaux variables, dans toutes les cellules testées. L'inactivation de PTBP1 par ARN interférence dans ces mêmes cellules n'a eu aucun effet sur l'abondance des ARN circulaires telle qu'évaluée par une amplification de la région d'épissage rétrograde. Avec ce type d'analyse, il nous est cependant impossible de conclure quant à la bonne circularisation de ces ARNs qui pourrait faire l'objet d'analyses ultérieures plus complexes, en particulier par Northern Blot afin de permettre la visualisation des molécules pleine longueur.

IV. Conclusion et perspectives ouvertes par ce travail

En résumé, au cours de ce projet de thèse nous avons réussi à identifier le premier régulateur de l'épissage alternatif de *TP63* : la PTBP1. Cette régulation conditionne la production de l'isoforme γ vis-à-vis des autres isoformes plus longues. Il s'agit d'un mécanisme de régulation qui est au moins conservé du Xénope jusqu'à l'homme, dont la divergence remonte à près de 400 millions d'années. L'isoforme γ , ainsi que la régulation de sa production, sont donc fortement conservés, ce qui suggère une importance fonctionnelle suffisante à assurer une pression de sélection. Ce mécanisme est également conservé dans les cellules de carcinomes épidermoïdes de la tête et du cou, où *TP63* est fréquemment amplifié et/ou surexprimé. Nous avons identifié une corrélation négative entre l'abondance de *TP63* γ et la survie de patients atteints de carcinomes épidermoïdes de la tête et du cou.

Dans des données d'expression tissulaires, l'isoforme γ est généralement très peu exprimée dans les différents tissus en comparaison à l'isoforme α , ceci à l'exception du muscle où elle est l'isoforme dominante. P63 est connu pour son rôle en tant que facteur de transcription, pour autant son expression musculaire semble être uniquement cytoplasmique ce qui suggère une fonction autre que transcriptionnelle. L'isoforme γ pourrait donc avoir une fonction tissu-spécifique restreinte aux muscles. Compte tenu de l'importance de p63 au cours du développement, il est également possible qu'elle ait une fenêtre temporelle d'utilité dans ce contexte. Dans les cellules épithéliales, il a été suggéré qu'elle pouvait promouvoir la transition épithélio-mésenchymateuse. Nous n'avons pas été capables d'étayer cette piste.

Des anticorps spécifiques de p63 γ seraient d'une aide cruciale dans l'analyse de ses fonctions protéiques, γ compris sa localisation subcellulaire et au sein de complexes

multimériques de p63. Bien que nous ayons réussi à développer des anticorps avec une bonne sensibilité et spécificité pour la protéine p63 surexprimée, ils restent à améliorer avant d'être utilisables dans l'analyse de p63 endogène. L'existence d'ARN circulaires de *TP63* dans les cellules HNSCC offre également une nouvelle piste d'investigation de ses fonctions non-protéiques dans ce type de cancer.

Le gène *TP63* permet la production de multiples espèces moléculaires, ARNm, isoformes protéiques et ARN circulaires qui peuvent potentiellement toutes moduler la fonction cellulaire. La régulation de la production de ces différents acteurs semble importante puisque la structure génique de *TP63* est fortement conservée. Déterminer quelle part prend chacun de ces acteurs dans le maintien d'un fonctionnement cellulaire normal s'avère une gageure qu'il conviendra d'aborder dans l'objectif d'élucider les fonctions de *TP63* dans des contextes pathologiques pouvant être aussi bien associés à la carcinogenèse qu'aux problèmes de développement.

Titre : Régulation post-transcriptionnelle des isoformes de *TP63* par la PTBP1 dans les carcinomes épidermoïdes de la tête et du cou

Mots clés : famille p53, épissage alternatif, biomarqueurs, RNABP, EMT.

Résumé : La protéine tumorale p63 (p63) codée par le gène *TP63* est un facteur de transcription de la famille de p53. *TP63* est essentiel au développement embryonnaire, son absence empêche la mise en place de la peau et s'accompagne de malformations voire d'absence totale des membres supérieurs et de défauts de morphogenèse craniofaciale. Au niveau cellulaire, *TP63* est impliqué dans le renouvellement des cellules souches, la différenciation et la prolifération cellulaire, la promotion de la transition épithélio-mésenchymateuse ou bien encore le maintien de l'intégrité de la lignée germinale. Des promoteurs alternatifs et plusieurs événements d'épissage alternatif conduisent à la production d'au moins huit isoformes de p63. Côté C-terminal, on distingue quatre principales isoformes : α , β , γ et δ . L'existence de multiples isoformes est souvent utilisée pour expliquer la complexité des événements cellulaires dépendant de *TP63*.

TP63 est fréquemment amplifié et/ou surexprimé dans les carcinomes épidermoïdes et nous avons mis en évidence une corrélation négative entre le niveau d'expression des isoformes minoritaires γ et la survie des patients atteints de carcinomes épidermoïdes de la tête et du cou. Par une combinaison d'approches bioinformatique et d'analyses mécanistiques, j'ai pu mettre en évidence qu'une interaction directe entre la protéine de liaison aux ARN PTBP1 et l'ARN pré-messager de *TP63* réprime l'utilisation de l'exon terminal γ dans un contexte de lignées cellulaires issues de carcinomes épidermoïdes. L'importance fonctionnelle de cette régulation est supportée par sa conservation des amphibiens à l'homme. Toutefois, les conséquences fonctionnelles d'une modification de l'abondance des isoformes minoritaires de p63 demeure une question en suspens.

Title : Post-transcriptional control of *TP63* isoforms by PTBP1 in Head and Neck Squamous Cell Carcinoma.

Keywords : p53 family, alternative splicing, biomarkers, RNABP, EMT

Abstract : The tumour protein p63 (p63), encoded by the *TP63* gene, is a transcription factor belonging to the p53 family. *TP63* is essential for embryonic development, its inactivation leading to defects in skin, limb and cranio-facial development. In cells, *TP63* is involved in stem cell renewal, differentiation, proliferation, control of epithelial to mesenchymal transition, and in the control of the genomic integrity of germ cells. Alternative promoters and alternative splicing events allow for the production of at least eight different p63 protein isoforms. At the C-terminal end, α , β , γ and δ isoforms differ by the occurrence of functional protein domains involved in the control of the activity or lifespan of the protein. The shorter γ isoform is produced by the use of a distinct alternative 3'UTR. The existence of multiple isoforms is often used to explain the many and sometimes conflicting roles of *TP63* in cellular phenotypes.

TP63 is often amplified or overexpressed in squamous cell carcinoma and we found a negative correlation between the γ isoform and Head and Neck Squamous Cell Carcinoma patient survival. By combining bioinformatic analysis of alternative splicing data and mechanistic analysis of *TP63* alternative splicing, I identified a direct binding between the splicing regulator PTBP1 and *TP63* pre-mRNA that controls the outcome of *TP63* gene expression by repressing the production of the γ terminal exon in squamous cell carcinoma cell lines. This is the first identification of a transacting factor controlling *TP63* splicing. The functional importance of this regulation is further supported by its conservation from Amphibians to Humans. However, questions concerning the functional consequences of changes in the abundance of the γ isoform remain open.