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WRAP53 UNWRAPPED; ROLES IN NUCLEAR ARCHITECTURE AND CANCER

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WRAP53 unwrapped; roles in nuclear architecture and cancer

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To my beloved Diana and Family

ABSTRACT

WRAP53 is a gene of multiple functions; it encodes for a natural antisense transcript that regulates the actions of the tumour suppressor p53, and it gives rise to a protein with oncogenic properties that is important for Cajal body formation and maintenance. Natural antisense transcripts are a group of regulatory RNAs implicated in many aspects of eukaryotic gene expression including transcription, RNA localization, translation and RNA stability. Even though up to 70% of all human genes may overlap in an antisense fashion, little is known about their biological significance. In this work, we have identified a natural antisense transcript of p53 that regulates the steady-state levels of endogenous p53 mRNA and the induction of p53 in response to DNA damage. p53 is a transcription factor that upon DNA damage and other types of cellular stress induces either growth arrest or apoptosis. The significance of p53 in cancer is clearly demonstrated by the fact that approximately 50% of human cancers carry alterations within the p53 gene and that the remaining tumours frequently show other defects within the p53 pathway. Our discovery that WRAP53 stabilizes the p53 mRNA by targeting its 5' untranslated region, reveals a novel pathway for p53 regulation and also suggests a novel therapeutic strategy for cancer treatment.

The *WRAP53* gene also codes for a protein that has a major role in Cajal body integrity and function. Cajal bodies are nuclear structures implicated in diverse functions such as maturation of the splicing machinery and telomere biogenesis. The WRAP53 protein has been shown to direct small Cajal body-specific RNAs, including the TERT, the RNA part of the telomerase complex, to these nuclear structures. We reveal that WRAP53 in addition directs the survival of motor neuron (SMN) complex to Cajal bodies and that WRAP53 is essential for Cajal body integrity. Our findings further highlight the role of WRAP53 as a Cajal body recruitment factor and contribute to our understanding of the molecular mechanisms behind Cajal body formation.

Interestingly, we also find that WRAP53 has oncogenic properties and is overexpressed in human cancers in comparison to primary cells. Moreover, knockdown of WRAP53 leads to massive apoptosis through the intrinsic mitochondrial pathway. Human cancer cells are more sensitive to WRAP53 depletion as compared to normal human fibroblasts, identifying WRAP53 as a novel therapeutic target in cancer. Altogether, our findings suggest an important role of the WRAP53 RNA and protein in tumourigenesis with great implications in cancer therapy.

ORIGINAL PUBLICATIONS

The thesis is based on the following papers, which in the text will be referred to by their roman numerals (I-III)

I. **Mahmoudi S.**, Henriksson S., Corcoran M., Méndez-Vidal C., Wiman KG. and Farnebo M., Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage, Molecular Cell, 2009, Feb 27;33(4):462-71

II. **Mahmoudi S**, Henriksson S, Weibrecht I, Smith S, Söderberg O, Strömblad S, Wiman KG and Farnebo M, WRAP53 is Essential for Cajal Body Formation and for Targeting the SMN Complex to Cajal Bodies, PLoS Biology, 2010, Nov 2;8(11):e1000521

III. **Mahmoudi S***, Henriksson S*, Farnebo L, Roberg K and Farnebo M, WRAP53 promotes cancer cell survival and is a potential target for cancer therapy, Cell Death and Disease, *In Press*

*Both authors contributed equally

RELATED PUBLICATIONS

I. Vilborg A, Glahder JA, Wilhelm MT, Bersani C, Corcoran M, **Mahmoudi S**, Rosenstierne M, Grandér D, Farnebo M, Norrild B, Wiman KG. The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element. Proc Natl Acad Sci U S A. 2009, Sep 15;106(37):15756-61

II. Karimi M, Conserva F, **Mahmoudi S**, Bergman J, Wiman KG, Bykov VJ. Extract from Asteraceae Brachylaena ramiflora induces apoptosis preferentially in mutant p53-expressing human tumor cells. Carcinogenesis. 2010, Jun;31(6):1045-53

III. Ullén A, Farnebo M, Thyrell L, **Mahmoudi S**, Kharaziha P, Lennartsson L, Grandér D, Panaretakis T, Nilsson S. Sorafenib induces apoptosis and autophagy in prostate cancer cells in vitro. Int J Oncol., 2010, Jul;37(1):15-20

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1. LIST OF ABBREVIATIONS

5'm7G	5'trimethylguanosine cap
AIF	Apoptosis inducing factor
ALT	Alternative lengthening of telomeres
APAF1	Apoptotic protease activating factor 1
ARE	Adenosine/Uracil (AU) rich elements
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Bak	Bcl-2 homologues antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-6	B-cell lymphoma 6
Bcl-XL	Bcl-2 relayed gene, long isoform
CAB box	Cajal body box
CDK	Cyclin-dependent kinase
CDK2	Cyclin-dependent kinase 2
Cyt C	Cytochrome C
DDR	DNA damage response
DR5	Death receptor 5
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
endo-siRNA	endogenous siRNA
FADD	Fas-associated death domain
G2/M	Gap 2/Mitosis
gems	gemini of coiled bodies
gRNA	Guide RNA
HLB	Histone locus body
HuR	Human antigen R
IF	Immunofluorescence
In situ PLA	*In situ* proximity ligation assay
IP	Immunoprecipitation
IRES	Internal ribosomal entry site
kDa	Kilo Dalton
lncRNA	Long non-coding RNA
lincRNAs	Long intergenic non-coding RNA
Lsm	Like Sm

Mdm2	Mouse double minute 2
mRNA	Messenger RNA
NAT	Natural antisense transcript
ncRNA	Non-coding RNA
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PHAX	Phosphorylated adaptor for RNA export
PKCδ	Protein kinase C, delta
PML	Promyelocytic leukemia
Poly(A)	Poly adenosine
PUMA	P53 upregulated modulator of apoptosis
RISC?	RNA-induced silencing complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPL26	60S ribosomal protein L26
rRNA	ribosomal RNA
siRNA	Small interfering RNA
scaRNA	Small Cajal body-specific RNA
Sm	Smith antigen
SMA	Spinal muscular atrophy
SMN	Survival of motor neurons protein
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
ssRNA	Single stranded RNA
TCAB-1	Telomerase Cajal body protein 1
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGS-1	Trimethylguanosine synthase 1
TMG	Trimethylguanosine
tRNA	Transfer RNA
U1-12	Uracil-rich snRNA1-12
Unrip	Unr (upstream of N-ras) interacting protein
UTR	Untranslated region
Wdr79	WD40 repeat protein 79
Wig-1	Wild type p53 induced gene 1
WRAP53	WD40 encoding RNA Antisense to p53
ZEB2	Zinc finger E-box-binding homeobox 2

2. INTRODUCTION

2.1 FOREWORD

This thesis touches upon many aspects of cell biology, including regulatory RNAs, tumour suppressors and oncogenes, cancer, cell death, splicing and nuclear structure formation and function. The reason is that WRAP53 is a multifaceted gene; it gives rise to a regulatory RNA that is important for the action of the tumour suppressor gene p53, and it also encodes for a protein with cancer promoting properties that is essential for the formation and maintenance of specific nuclear structures called Cajal bodies. Although at first it may be a little difficult to imagine how all these parts relate to each other, I hope that as the thesis progresses you might appreciate how nature has elegantly constructed this gene to fit its needs. This work is just the beginning, and future studies will certainly shed more light on the apparently complex genomic arrangement of the WRAP53 gene and its biological significance. But that I will leave to my fellow colleagues.

With that said let's take a closer look at the biology of cancer.

2.2 IT IS ALL ABOUT BALANCE

A healthy life is all about balance. You need to find a balance between work and personal life, and between eating and exercising to remain in good psychological and physical shape. The same rule applies to your organs and tissues; they need to coordinate the balance between cells that are growing and the cells that are dying for the well-being of the tissues. Imbalance in either or both of these processes may have dire consequences causing hyperproliferative diseases such as cancer. Considering the importance of sustaining tissue homeostasis, the cell has developed an arsenal of regulatory circuits to be able to make the decision on whether it should keep on growing and dividing, or arrest and die. This decision is based on its environment as well as the inner condition of the cell that continuously signals to a group of regulators of life or death that will in turn interpret these signals to execute appropriate responses. This important repertoire of cellular moderators can be divided into two groups; oncogenes and tumour suppressor genes. While oncogenes are the green lights of the cell, promoting growth and preventing cell death, tumour suppressors are the red lights of the cell, preventing proliferation and inducing arrest and cell death. Malfunction in one or several of these essential regulatory proteins is enough to initiate a process that eventually can lead to the growth of a tumour. Considering the large number of existing oncogenes and tumour suppressors, a variety of different combinations can induce tumour development. Thus cancer is not one disease but represents a spectrum of different diseases, each with their own set of genetic defects. Nevertheless, all cancers have some characteristic phenotypes in common. They all have rendered themselves insensitive to growth suppressive and apoptotic factors by inactivating tumour suppressor genes. In addition, they have activated proto-oncogenes to be able to keep on proliferating, producing new blood vessels to maintain the constant flow of

nutrients and oxygen, and have acquired invasive properties to be able to move out from their own niche[1]. All these changes disrupt the sacred equilibrium in tissue homeostasis in one way or another. Thus, cancer can simply be described as a disease where the cell has gone out of balance.

What is then an antisense transcript and how is that related to p53 regulation? Well, to understand that we need to take one step back and see what has happened in the biological research community the past decade.

2.3 REVISING OUR THOUGHTS

No area of molecular biology has experienced a more dramatic change in concept and perspective the past decade than the ribonucleic acid (RNA) field. With that, also the traditional view of a gene has been revised. A gene was considered to be a segment of DNA giving rise to the functional end product: a protein. The principal role of the RNA was to transfer the genetic information from the DNA to the protein, either as a messenger RNA (mRNA) or as part of the translational machinery in the form of ribosomal (r)RNA and transfer (t)RNA. This view has rapidly changed in recent years, in part by data from large-scale transcriptome projects and in part by the constant identification of new classes of regulatory RNAs. The fact that 80% of the human genome is being transcribed while only 2% of the genome encodes for proteins suggests that these transcripts are more than transcriptional noise[2,3,4,5]. Indeed, the past decade has shown us that RNAs *per se* can have similar functional capabilities as cellular proteins. Today we know that a great number of RNAs play essential roles in many biological processes, such as in regulation of gene expression (microRNAs, small interfering (si)RNAs, promoter-associated RNAs, natural antisense transcripts(NATs)), RNA processing and modification (small nuclear (sn)RNAs, small nucleolar (sno)RNAs, small Cajal body (sca)RNAs), editing (guide (g)RNAs), protein export (7S RNA in signal recognition particle), genomic stability (telomerase RNA, TERC), catalytic processes (ribozymes), epigenetics (long intergenic non-coding (linc)RNAs), and the list goes on. As the technology improves and becomes more cost-effective – researchers are discovering that there is an RNA world out there. Thus, we are revising what we used to consider junk DNA and functional genes.

For the purpose of this thesis I will focus on only two types of regulatory RNAs, namely the NATs and the RNAs involved in RNA processing and modification. Lets start by taking a closer look at the NAT field.

2.4 NATURAL ANTISENSE TRANSCRIPTS

A transcriptional jungle is emerging in which both DNA strands are simultaneously transcribed, genes overlap each other in both orientations and many transcripts with essential biological functions do not code for proteins. NATs are a group of regulatory RNAs that are defined by their complete or partial complementarity to target (sense) RNAs. They are divided into two

groups depending on whether they act in *trans* or *cis*. While *trans*-NATs are transcribed from different locations than their targets such as microRNAs, *cis*-NATs are transcribed from the same genomic locus as their target but from the opposite DNA strand.

Large-scale transcriptome projects indicate that overlapping genes are a common phenomenon in the human genome. Up to 50-70% of all genes have been suggested to have antisense partners[6,7,8,9]. They can overlap in a head-to-head (5' to 5') or tail-to-tail (3' to 3') direction or one gene may completely overlap the other gene (Figure 1). Many antisense transcripts have maintained their genomic organization during evolution suggesting that they have a functional significance[10]. Although the majority of these transcripts are non-coding RNAs[8], there are many examples of protein-coding genes with overlaps[11,12]. Expression profiling as well as experimental studies have revealed that the sense/antisense pairs may be expressed in a concordant or discordant fashion. While a concordant expression describes a positive correlation between the sense/antisense pair – knockdown of antisense lead to decreased levels of sense transcript, the discordant regulation refers to an inverse correlation – knockdown of antisense leads to increased levels of the sense transcript[8,13]. Although only a handful of sense/antisense pairs have been carefully characterized[11,14,15], it has been suggested that antisense regulation is a common type of gene control in the human genome.

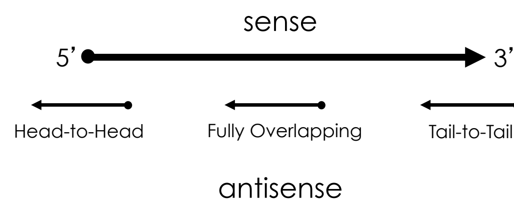

Figure 1: Illustration of the different types of overlap between sense/antisense genes

2.4.1 Mechanisms of Action

NATs are a heterogeneous group of RNAs, apparent from the fact that they display both concordant and discordant types of regulation. Several models have been proposed to explain the molecular mechanisms of *cis*-acting NATs, including transcriptional interference, RNA masking, epigenetic alteration and double-stranded RNA (dsRNA) mechanisms[16,17]. Next will follow a short description of each model and, when possible, an example with implications in cancer.

2.4.1.1 Transcriptional Interference

The transcriptional collision model was originally suggested from studies in yeast, where researchers observed that as the length of the overlapping region increases, the expression level of *cis*-NATs decreases[18,19,20]. Using atomic force microscopy on convergent transcription complexes in *E.coli* [21], it was recently shown that as RNA polymerases approach each other, they are not able

to either pass or displace one another, instead they stop at the crash site. This leads to transcriptional interference and the degradation of the incomplete transcripts. In this type of regulation, it is not the antisense transcript *per se* that is important for the regulation but rather the act of transcription. I*n silico* studies suggest that transcriptional collision might also be prevalent in mammals, especially for sense/antisense genes with an overlap of more than 2 kb[22]. However, it is not believed to be the predominant mechanisms of action since transcription can occur at different times at the same chromosomal locus or sense/antisense genes may be transcribed at the same time from different chromosomes[16].

2.4.1.2 RNA Masking

RNA masking refers to the function of an antisense transcript to block splice sites or block binding of enhancers/repressors of splicing. In this way the antisense transcript will change the balance between splice variants, favouring one splice variant over the other. An example of this, which also has implications in cancer, is the antisense gene of ZEB2/SIP1 called ZEB2 AS[23]. ZEB2 is a transcriptional repressor of E-cadherin, a cell-cell adhesion protein whose downregulation is associated with increased proliferation, invasion and/or metastasis[24]. The ZEB2 AS inhibits the splicing of the first intron of ZEB2, favouring a splice variant with enhanced translational capacity. This leads to increased levels of ZEB2 and consequently decreased levels of E-cadherin[23]. ZEB2 AS is often overexpressed in tumours with low E-cadherin expression[23].

2.4.1.3 Epigenetic Modulators

NATs have also been linked to epigenetic regulation via DNA methylation or chromatin remodelling [25,26,27,28]. Antisense transcripts have been suggested to recruit either DNA or histone-modifying enzymes, leading to epigenetic changes[16,29]. Depending on the type of complex that the NAT recruits, it can hypothetically lead to increased or decreased transcription of the sense mRNA. The antisense transcripts of both the tumour suppressor genes p15 and p21 have been shown to recruit chromatin remodelling complexes that induce inhibitory histone modifications to shut down their transcription[26,28].

2.4.1.4 dsRNA Mechanisms

dsRNA duplex formation, which refers to the interaction between the sense and antisense transcript, may have several outcomes leading to both concordant and discordant regulation. Sense/antisense duplex formation has been suggested to lead to RNA editing, activation of the RNAi pathway, and changes in RNA turnover, export and translation rates[16].

RNA editing refers to numerous cellular processes that change the RNA sequence from that designated by their DNA templates[30]. One such process is the deamination of adenosine to inosine in dsRNA that can lead to nuclear retention or cytoplasmic degradation of the RNA transcripts[31]. Although, RNA editing has been shown to be prevalent in introns of primary transcripts, they are uncommon in sense/antisense overlapping regions[32], suggesting that it is not a common pathway of action.

Sense/antisense hybrids can also be targeted by the RNAi pathway to be processed into so-called endogenous small interfering RNAs (endo-siRNA), which would lead to the sequence-specific silencing of targeted genes. In fact, several endogenous siRNAs mapped to overlapping transcripts have been identified in Arabidopsis and Drosophila[33,34]. Their existence has also been confirmed in both mouse and human in recent years [35,36], suggesting a role of this pathway in NAT-mediated gene regulation.

Antisense transcripts can also directly or indirectly induce changes in the stability of their sense mRNAs[11,14,15,37]. They have been proposed to act by nuclear import/export mechanisms, by inducing alterations in the secondary structure of the sense transcript to either expose or mask binding sites for stabilizing/destabilizing factors, by masking microRNA-binding sites or by recruiting stabilizing factors[16]. WRAP53 belongs to this group of NATs, since it stabilizes p53 mRNA seemingly via direct RNA-RNA interaction[11]. WRAP53 will be discussed in detail in Paper I.

Clearly, cis-NATs can have impact on many levels of gene expression. As additional NATs are being identified and characterized also their roles in further biological settings are being uncovered. Future studies will certainly yield new insights into both their prevalence and significance in the human genome. Now it is time to turn our focus to another aspect of my thesis, namely the tumour suppressor gene p53, and to why p53 is one of the most studied genes in history, all categories.

2.5 THE GUARDIAN OF THE GENOME

One of the biggest obstacles for most cancers is to escape the tumour suppressor gene p53, also known as the guardian of the genome. This is demonstrated by the fact that the p53 gene is mutated in more than 50% of all human cancers and that the rest have in one way or another inactivated the p53 pathway[38](www.iarc.fr/p53; p53.free.fr). p53 is a transcription factor that is induced upon cellular stress, such as DNA damage, oncogene activation, nutrient deprivation, hypoxia, and ribosomal stress. Depending on the type, strength and persistence of the stress, p53 will alter the expression of a subset of target genes to induce cell cycle arrest, apoptosis, senescence, differentiation, DNA repair, inhibition of angiogenesis and even survival [39,40,41,42,43]. However, the function of p53 goes beyond its function in stress response, p53 has been implicated in a wide range of processes such as embryonic development, neurodegenerative and metabolic diseases and aging[44,45,46].

p53 acts at several levels to direct the cell towards the desired response. Its role as a transcriptional activator is probably the best characterized function of p53. The p53 protein forms a tetramer that binds DNA with a certain consensus site, which is made up of two tandem copies of the motif "RRRCWWGYYY" separated by a spacer of 0 to 13 base pairs ("R" represents purines, "W" represents adenine or thymine, and "Y" represents pyrimidines). By binding to the DNA, p53 recruits the general transcription machinery to the promoter-enhancer region of its target genes, inducing their transcription[47,48]. However, p53 can also block transcription by binding to DNA[49]. In addition, p53 can re-localize to the

mitochondria and directly promote apoptosis[50] as well as affect RNA-binding and microRNA processing[51].

The list of p53 target genes is long and it keeps growing, adding new potential outcomes of p53 induction. But how exactly does p53 decide which genes to activate? Well, there is no simple answer to this question but there are several hypothetical explanations. One explanation refers to the affinity of the p53 protein to its target genes. While some genes have perfect p53 consensus sites (high affinity), other genes have less perfect but still functional sites (low-affinity). Since cell cycle genes tend to have high affinity response elements, whereas pro-apoptotic genes have low-affinity response elements, the initial p53 response is cell cycle arrest followed by apoptosis. The abundance of the p53 protein also plays an important role in p53's choice of targets, where high levels of p53 promote a pro-apoptotic response. In addition, post-transcriptional modifications as well as cofactors bound to the p53 protein affect p53's selectivity [40,52,53,54]. Thus, the abundance of the p53 protein, the persistence of its activation, post-translational modifications and p53 binding partners help p53 in the activation of the appropriate response. Moreover, basal levels of p53 are enough to execute certain of its functions such as those involved in fertility, development, metabolism, and stem cell maintenance[53,55].

2.5.1 p53 in DNA Damage Response

DNA damage has many origins; it can be induced by exposure to different environmental stress factors such as radiation or DNA damaging agents or it can occur during natural processes such as mitosis or DNA replication. Since DNA damage is practically inevitable, eukaryotes have developed a highly conserved genome surveillance system called the DNA damage response (DDR). Depending on the type of DNA damage, DDR will activate different signalling cascades that will initially induce cell cycle arrest, allowing the cell to repair the damage, and if the damage persists to trigger apoptosis. p53 is a central player in this process. From being barely detectable in healthy non-stressed cells, p53 levels are dramatically increased upon DNA damage. This induction is mainly achieved by the stabilization of the p53 protein that allows p53 to exert its function as a transcription factor [54,56]. Interestingly, p53 has been shown to be activated in a cyclically periodic manner upon DNA damage[57]. This would provide the cell time to repair the DNA damage during the first activation and if the damage persists to induce cell death upon the second round of induction[56].

An important target gene in the induction of cell cycle arrests is p21. p21 is a cyclin-dependent kinase (CDK) inhibitor that upon induction binds and blocks cyclin-CDK complexes. Cyclin-CDK complexes are essential in the progression of the cell-cycle and once blocked the cell-cycle will halt to allow for DNA repair. In addition to inhibiting cyclin-CDK complexes, p21 associates with PCNA, a protein involved in DNA synthesis, to block DNA replication[58].

If the damage is too severe for the cell to handle, the p53 response shifts to apoptosis. P53 can induce both mitochondrial-independent cell death via induction of the death receptor Fas, as well as mitochondrial-dependent

apoptosis via the BH3-family proteins Bax, NOXA and PUMA[59]. I will cover the different cell death pathways later on so bear with me for now.

2.5.2 p53 Regulation

Considering that p53, literally, decides over life and death, the cell needs to keep a close eye on p53 levels. You do not want to die if you do not need to, right? Thus different regulatory mechanisms have been established to ensure that p53 gets activated only when necessary, and to control that p53 induces the right response to a given signal[60,61].

Thirty years of intense p53 research have revealed an impressive arsenal of regulatory circuits that can induce p53 response. However, the focus has been mainly, if not solely, on the post-translational regulation of p53. Relatively little is known about the transcriptional and post-transcriptional regulation of p53. It is only until quite recently that just a handful of papers have been published regarding this aspect of p53 regulation, in comparison to thousands that deal with its post-translational regulation.

2.5.2.1 Post-translational Regulation

To continuously synthesize and degrade a protein is an efficient way for a cell to maintain an important regulator of life and death at low levels while allowing it to have a rapid response to a stimulus. Thus, the p53 protein is degraded almost as soon as it has been synthesized in normal, healthy cells. There are multiple mechanisms that keep p53 at low levels but the main regulator is Mdm2. Mdm2 is an E3 ligase that targets p53 for degradation by adding ubiquitin proteins to it, in a process called poly-ubiquitination. The addition of these small molecules marks p53 as a substrate for the cell's protein degradation machinery, the proteasome. Mdm2 also binds to p53 to mask its transactivation domain, preventing p53 from activating target genes. To ensure that the p53 induction upon stress is not stronger or persists longer than necessary, Mdm2 is also a target gene of p53. Thus, there is a negative feed-back loop where induction of p53 leads to its own destruction by increasing the levels of its E3 ligase, Mdm2. In addition to Mdm2, there are many other proteins shown to bind and regulate p53, and p53 activity and action can also be regulated by a number of post-translational modifications such as phosphorylation, acetylation and methylation[62,63].

2.5.2.2 Transcriptional Regulation

Little is known about the transcriptional regulation of p53. The few studies conducted suggest that p53 in fact can transactivatate itself[64] and also that PKCδ and HOXA5 are positive and Bcl6 negative regulators of p53 transcription[65,66,67].

As I mentioned earlier, the constant synthesis and degradation of a protein might allow for a rapid response to external stimuli but it is also very energy consuming. A slightly slower but more energy-conserving way of regulating gene expression is via post-transcriptional pathways, which include modification of mRNA stability or translational rates. Before I get into the post-transcriptional regulation of p53, lets take a moment to discuss the mRNA per se.

2.5.2.3 The Conventional mRNA

A conventional eukaryotic mRNA encodes for a protein and is synthesized by RNA polymerase II. Immediately after transcription, the mRNA is capped with a 5'7-methylguanosine structure and a 3'poly(A) tail is added. These two structures are essential for the processing of the mRNA and provide binding sites for different types of factors affecting export, translation and stability of the mRNA transcript[68]. Before the transcript is shipped to the cytoplasm for translation of the protein, the non-coding intervening sequences called introns must be removed so that the mRNA can be translated into the correct protein. The removal of introns and rejoining of the exons is called splicing and will be discussed later in the thesis.

The mRNA *per se* is divided into three regions; a 5' untranslated region (UTR), an open reading frame (ORF) and a 3'UTR. While the ORF codes for the protein, there are sequences within the two UTRs that control different aspects of mRNA regulation such as stability/turnover, export, silencing and translation. Examples of regulatory sequences are internal ribosomal entry sites (IRES), which provide alternative translation initiations sites, AU-rich elements (AREs), which provide docking sites usually for degradation and translational inhibition factors, microRNA sites for Drosha-mediated regulation as well as protein-binding sequences[69,70,71].

2.5.2.4 The Importance of mRNA Stability

mRNA turnover is important for the abundance of cellular transcripts and the proteins they encode[72]. While some mRNAs are degraded within a few hours other transcripts have half-lives extending over several cell cycles[73]. The decay rates of most transcripts, such as those encoding housekeeping genes, do not change upon environmental signals[68]. However, transcripts encoding for proteins that need to be under tight control because of their important roles in, for example, cell growth or cell death, such as cyclins, p21 and p53, do change in response to external factors[68,74,75]. By providing stability and consequently changing decay rates of unstable mRNAs, the cell can in a rapid and energy-preserving way implement dramatic changes in the expression of specific genes[68].

There are several mRNA decay pathways initiated either from the 5' or the 3' ends. In general, the removal of the 5'cap or the poly(A) tail is rate limiting for degradation. These protective structures need to be removed for the exonucleases to access the mRNA ends. In mammals the main pathway of mRNA decay is via deadenylation. This happens either by the removal of the 5'cap by the exonuclease Xrn1 or the loss of the poly(A)-tail by the exosome. Although, most of the mRNA degradation occurs in the cytoplasm, there is evidence of the existence of the exosome in the nucleus[76].

2.5.3.5 Post-transcriptional Regulation

A series of papers published within the past few years have highlighted the importance of post-transcriptional regulation in p53 expression and function (Figure 2)[77]. All three regions of the p53 mRNA, including the 5'UTR, ORF and 3'UTR, have been implicated in the post-translational regulation. The existence of destabilizing elements in the 5'UTR of p53 was already suggested a decade

ago, when studies in chicken and mice showed that 5'UTR deletion constructs of p53 were significantly more stable compared to full-length p53 constructs[78]. In recent years, it has been shown that the p53 5'UTR can form a stem-loop which hinders ribosome access during cap-dependent translation initiation. The ribosomal protein RPL26 can help to overcome this inhibitory effect by promoting the usage of an IRES that exists within the 5'UTR[79]. In addition, the p53 3'UTR can fold back on itself and form a loop by base-pairing with a region in the 5'UTR, which further enhances the translational promoting effects of RPL26[80]. Nucleolin, another regulatory protein that was shown to bind the 5'UTR of p53, instead inhibits translation rates presumably by stabilizing the 5' stem-loop[79]. Furthermore, WRAP53, a *cis*-antisense gene of p53, stabilizes p53 mRNA via the 5'UTR of the mRNA [11]. The *WRAP53* gene and its function in p53 action are described in Paper I.

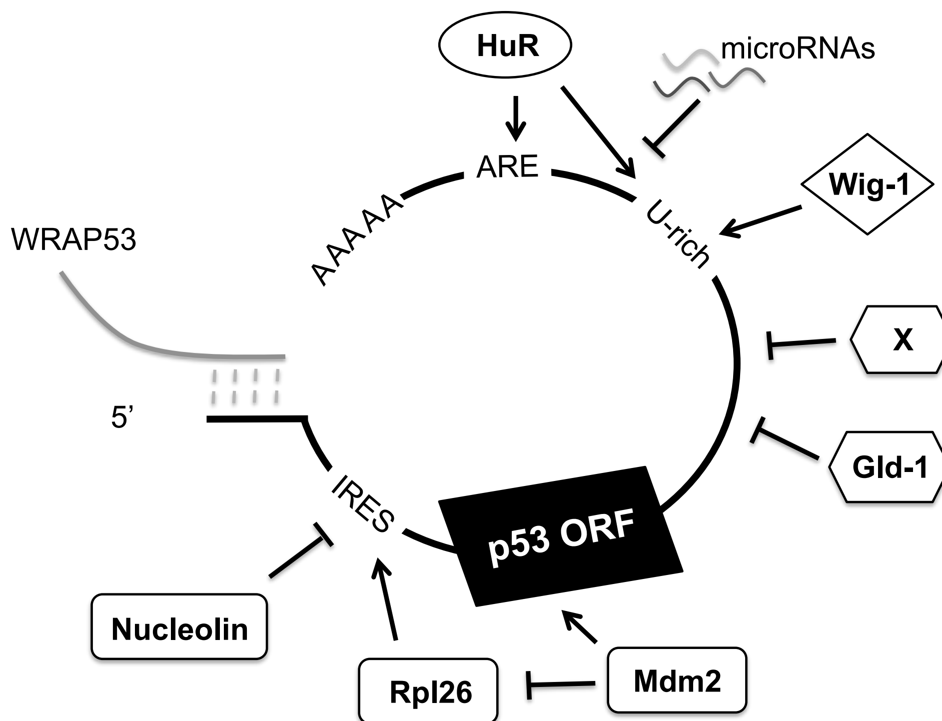

Figure 2: p53 mRNA and the factors that regulates it.

In addition to its role in targeting p53 for proteosomal degradation, Mdm2 has also been shown inhibit p53 translation by targeting Rpl26 for degradation[81]. However, Mdm2 can also bind a region of the p53 ORF, which stimulates p53 translation and inhibits the E3 ligase activity of Mdm2[11,82]. Thus Mdm2 has contradictory roles; it can both degrade p53 protein by ubiquitination and enhance p53 protein synthesis by promoting translation. This demonstrates the complexity of p53 regulation and highlights the importance of multiple regulatory pathways that seemingly overlap but are nevertheless required to keep the levels of p53 in tight control.

As mentioned earlier, the 3'UTR are in general important regulatory regions containing both stabilizing and destabilizing sequences. The 3'UTR of p53 is no exception; it contains AREs and other protein-binding sequences as well as microRNA-target sites. AREs exist in transcripts that generally require very precise control of their expression, such as in the proto-oncogene myc and the cyclins[75]. These sequences are protein-binding sites that are often bound by factors that exert a negative effect on the mRNA, by either promoting degradation or inhibiting translation. However, in the case p53, the ARE is bound by two positive regulators, namely HuR and Wig-1, which confer both increased p53 mRNA stability and increased translation[83,84,85,86]. Interestingly, Wig-1 is also a target gene of p53 that is induced upon stress, constituting a positive feedback loop to stabilize p53 mRNA levels upon DNA damage. This stresses the importance of increased p53 mRNA stability in DNA damage response. Also, two negative regulators of p53 translation have been reported to bind within the 3'UTR of p53, namely a still unidentified protein referred to as protein X and Gld-1[84,87]. Several microRNAs have also recently been found to regulate p53 expression by targeting the 3'UTR[88,89,90,91,92].

Another aspect of my thesis that is partly touched upon in Paper I, as part of the p53 DNA damage response, but that is the main focus of Paper III is what awaits all of us sooner or later: the eternal death!

2.6 DEATH AS PART OF LIFE

Every day 50-70 billion cells die in an adult human body. The maintenance of cell numbers and cellular positioning within the different tissues are prerequisites for the well-being of a multicellular organism. Defects in the regulation of cell death results in pathological conditions such as developmental defects, autoimmune and neurodegenerative diseases, and cancer[93,94,95].

There are two major types of cell death; programmed cell death, also known as apoptosis, and necrosis. Apoptosis is a programmed, multistep, energy-dependent cell death characterized by membrane blebbing, cell shrinkage, chromatin condensation and ultimately nuclear and cellular fragmentation into apoptotic bodies. Since neighbouring phagocytic cells engulf these nuclear bodies, their inner content is not released to the outer tissue and no immune response is triggered. Necrosis, on the other hand, occurs when cells rupture, often due to external factors such as heat or cold, mechanical tearing of cells and low blood supply. Necrotic cell death leads to the release of cellular compartments into the surrounding tissue, triggering an inflammatory reaction by the immune system[96,97,98].

2.6.1 Apoptosis

Programmed cell death is a highly conserved mechanism responsible for the removal of unwanted and damaged cells, which is critical for tissue homeostasis and development[99,100]. Two signalling pathways trigger apoptosis; the extrinsic or receptor-mediated pathway, and the intrinsic or mitochondrial-mediated pathway (Figure 3). While the extrinsic pathway has its origin outside

the cell through the activation of specific death receptors on the cell surface, the intrinsic pathway is initiated from within the cell[93,101].

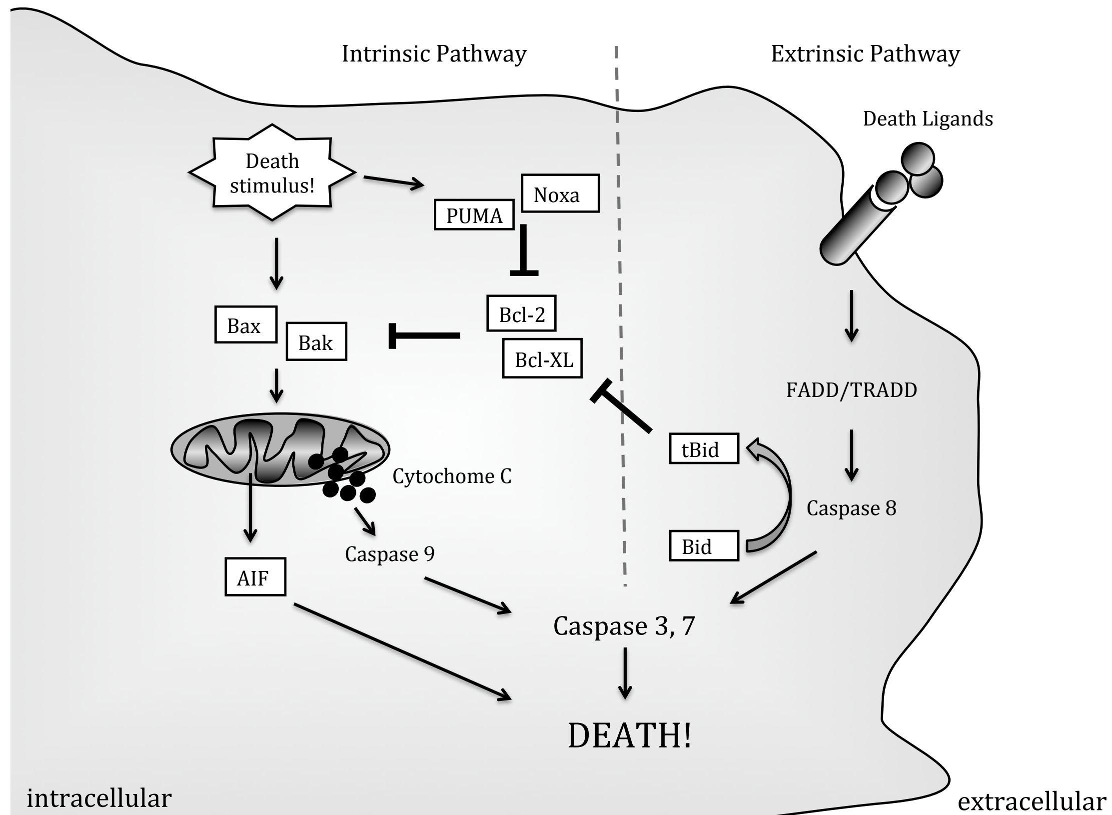

Figure 3: A simplified overview of the intrinsic and the extrinsic apoptotic pathways. tBid stands for truncated Bid

2.6.2 Caspases

Caspase (cysteine-dependent aspartate specific protease) activation plays a central role in both intrinsic and extrinsic pathways. In normal cells, all caspases exist in an inactive form (pro-caspase); however, upon death stimuli, these caspases are proteolytically cleaved, rendering them active. In general, they are divided into initiator and executioner caspases. Initiator caspases, such as caspase 8 and 9, are activated upon death stimuli by autocleavage and they will in turn cleave the executioner caspase, such as caspase 3, 6 and 7, to shift them into their active forms. The executioner caspases will then breakdown the cytoskeletal and nuclear structures as well as activate DNases that subsequently will fragment the DNA into small pieces[93,102,103].

2.6.3 The Mitochondria

Mitochondria, which are the major source of energy in all eukaryotic cells, are also the source of signals that initiate apoptotic cell death. They are composed of an outer and an inner mitochondria membrane, the integrity of both of which is important for the function of theses organelles. Disruption of the outer

membrane leads to the release of multiple proteins including cytochrome c (cyt c) from the inner membrane out to the cytosol[104,105]. The release of cyt c will promote the activation of the caspase 9, an initiator caspase that in turn will activate the executioner caspase 3[106]. Other pro-apoptotic factors that are also released from the disrupted mitochondria, includes SMAC/Diablo and AIF, which further promote cell death[107].

The permeability of the mitochondrial membrane is highly dependent on the balance between pro- and anti-apoptotic members of the Bcl-2 family[108,109]. The pro-apoptotic proteins Bax and/or Bak have been shown to form heterotetrameric channels that induce permeabilization of mitochondrial proteins such as cyt c[110]. The anti-apoptotic members, Bcl-2 and Bcl-XL, on the other hand, maintain the integrity of the membrane by inhibiting oligomerization of Bax and/or Bak[111]. The actions of Bcl-2/Bcl-XL can in turn be counteracted by other pro-apoptotic members such as PUMA, which binds Bcl-2/Bcl-XL and blocks their anti-apoptotic functions[112].

2.6.4 Death Receptors

The extrinsic pathway is triggered by the activation of the death receptors by their respective ligands, such as FasL binding to its receptor Fas. It can be induced by either the release of the death ligands or the upregulation of the death receptors to sensitize the cells to the existing ligands. Upon activation, a complex cascade of events is initiated that includes the recruitment of the adaptor protein Fas-associated death domain (FADD) and the formation of a death-inducing signalling complex (DISC) via caspase-8 or -10. The activation of DISC will in turn activate the effector caspases 3, 6, and 7, leading to fragmentation of the cell[93,113].

2.6.5 p53-Dependent and Independent Apoptosis

Both extrinsic and intrinsic pro-apoptotic genes are found among the extensive list of p53 target genes. p53-mediated apoptosis acts primarily through the intrinsic pathway by the transactivation of factors that act upstream of the mitochondria, such as Bax and PUMA, as well as genes that act downstream, such as APAF1. APAF1 acts as a coactivator of caspase 9 and helps to initiate the caspase cascade. However, p53 can also activate the extrinsic pathway by inducting the expression of Fas and DR5 death receptors as well as the gene coding for the Fas ligand, *TNFS6.* Furthermore, p53 can upregulate the expression of caspase 6 to potentiate the already initiated cell death[59,114,115].

Although p53 has a major role in the induction of the mitochondrial apoptotic pathway, the intrinsic pathway can also be induced by p53-independent means. In fact many of the genes that are strongly linked to p53-mediated apoptosis can also be triggered in the absence of p53[116,117,118]. An example is PUMA that is induced in response to a variety of signals such as ER stress and growth factor withdrawal[118].

It is time to completely shift gears and turn our attention to a specific nuclear structure called the Cajal body, which is the main focus of Paper II.

2.7 THE CAJAL BODY – A CENTURY LATER

The cell nucleus is highly organized and contains an array of nonmembrane-bound structures that allow for essential processes, such as RNA synthesis and processing, to occur in the most efficient way. The Cajal body is one of the organelles that was discovered in vertebrate neurons more than 100 years ago by the neurologist Santiago Ramon y Cajal[119]. It is an evolutionary conserved structure found in very divergent organisms including plants, insects, fish, mice and humans [120,121]. Cajal bodies are dynamic structures that move, split and fuse in the nucleoplasm and within the nucleolus[122] and their size and number varies depending on cell type and cell cycle stage[123]. Cajal bodies have been implicated in many biological processes such as in the spliceosomal snRNP biogenesis[124,125] and in the assembly of spliceosomal sub-complexes[126,127]. They have also been reported to be involved in the biogenesis and delivery of telomerase to telomeres[128,129,130,131]. Moreover, Cajal bodies contain basal transcription factors and can associate with snRNA genes, histone gene cluster and PML (promyelocytic leukemia) bodies[132,133,134]. Studies in Arabidopsis further suggest a role of Cajal bodies in certain steps of microRNA and siRNA biogenesis[135,136].

Although Cajal bodies have been linked to many essential processes in the cell, their biological significance remains unclear. Arabidopsis or Drosophila mutants lacking coilin (a signature protein of Cajal bodies described in detail below), and thus lacking Cajal bodies, are viable and show no obvious growth suppressive phenotype[137,138]. Similarly, human cells lacking Cajal bodies are viable but exhibit decreased proliferation and splicing rates[139,140]. However, coilin knockout mice display a semi-lethal phenotype. Half of these mice die at late gestation, and the remaining mice exhibit reduced fertility and fecundity[141]. In addition, knockdown of coilin in zebrafish is embryonically lethal[142]. Although these results may seem inconsistent, they have been suggested to reflect the differences in development rates between the organisms and their requirement for splicing efficiency[142]. Many of the processes that occur in the Cajal body can also take place in the nucleoplasm in the absence of Cajal bodies[143]. The formation of Cajal bodies is therefore believed to be a way for the cell to increase the efficiency of these processes by concentrating all the factors in one place. Thus, having Cajal bodies ensures robust and efficient processing during periods of rapid growth[144,145]. In contrast to this notion, they have also been suggested to regulate the rate of RNA processing in the cell by sequestering splicing components[146]. In either case, the fact that Cajal bodies are highly conserved in evolution provides evidence that they confer advantage to the organism.

2.7.1 Cajal Bodies and Cancer

Several lines of data suggest that Cajal bodies might be involved in carcinogenesis. Cajal body number and size are increased in transformed cells[147]. Although this may just be a reflection of the increased transcriptional/metabolic state of the cells, it also implies an increased need or even a dependency of Cajal body functions. Also, TERT has been shown to accumulate in Cajal bodies in cancer cells[148]. Furthermore, the region where

the coilin gene resides has been reported to be amplified in anaplastic, but not benign or atypical meningiomas[149]. Moreover, coilin -/- mice are less prone to develop spontaneous tumours[132]. Future studies will tell if these data are circumstantial or if Cajal bodies in fact have a role in tumour development. However, it is important to keep in mind that coilin has functions outside the Cajal bodies[150] (see below), suggesting that coilin *per se* may play a role in cancer progression.

Cajal bodies are enriched in a diversity of factors, such as coilin, snRNPs, SMN, snoRNPs, scaRNAs and TERT, reflecting the ongoing processes that take place in these structures. Lets go through the different components that may be of interest for us.

2.7.2 Coilin

Coilin is the marker protein for Cajal bodies and is essential for Cajal body integrity and function[151]. Although the exact function of coilin is not known, coilin is believed to act as the platform of the Cajal bodies and to bring all components of the Cajal body together to facilitate their various activities. Additionally, coilin has a role in the association of Cajal bodies with PML bodies[134], which are nuclear structures that are associated with transcriptional regulation, apoptosis and genome stability[152], and with gemini of coiled bodies (gems)[151] (described below). Coilin has an oligomerization domain in the N-terminus necessary for its localization to Cajal bodies[153], and Sm-protein and SMN-protein binding sites in its C-terminus [151,154]. Because coilin has separate binding domains for SMN and Sm, it has been suggested to interact with both free Sm-proteins and intact snRNPs[154], consequently having roles in snRNP biogenesis and recycling (these processes are described in more detail below).

Experimental removal of coilin leads to the disruption of typical Cajal bodies, whereas so called “residual Cajal bodies” remain. There are two types of residual Cajal bodies; one that contains scaRNAs [125] and one that contains proteins such as fibrillarin and Nopp140 [155]. However, neither of the structures is able to recruit SMN, highlighting the role of coilin in the association of the SMN complex with Cajal bodies. In line with these data, it has been suggested that Cajal bodies have separate compartments for different activities[140]. Inhibition of snRNP biogenesis by depletion of protein such as SMN, TGS1 and PHAX in HeLa cells, leads to the disruption of typical Cajal bodies, but at least two different coilin-containing nuclear bodies remain containing snoRNP/scaRNP components[140]. Therefore, it is conceivable that Cajal bodies indeed have different compartments and that depletion of factors in certain compartments only disrupts that compartment, while other compartments remain and form residual Cajal bodies.

The fact that only 30% of the total amount of coilin is found in the Cajal bodies[156], suggests that coilin has other functions in addition to its role in Cajal body formation and maintenance. Indeed, coilin has been recently implicated in DNA repair and centromere damage response[150,157].

snRNPs are the rate-limiting factors for de novo formation of Cajal bodies [140]. Let's have a brief overview of their function in splicing.

2.7.3 Splicing

RNA splicing refers to the process by which non-coding introns are removed from newly synthesised mRNA and the exons are rejoined. This is an essential process that occurs before a typical eukaryotic mRNA is exported to the cytoplasm for translation. The splicing reaction is performed in a series of reactions that are catalysed by the spliceosome, a large RNA/protein macromolecular machine. Spliceosomes consist of several ribonucleoprotein (RNP) units called snRNPs and numerous non-snRNP factors. A snRNP unit contains seven core proteins assembled on a uridine-rich RNA molecule called a U snRNA (described in more detail below)[158,159,160]. There are two types of spliceosomes consisting of slightly different snRNPs: the major and the minor spliceosomes. The major spliceosome is composed of U1, U2, U4, U5 and U6 snRNPs and is responsible for splicing of the vast majority of pre-mRNA introns[161]. The minor spliceosome consists of U11, U12, $U4_{ATAC}$, U5 (same as in the major form) and $U6_{ATAC}$ snRNPs and splices out rare classes of introns [162].

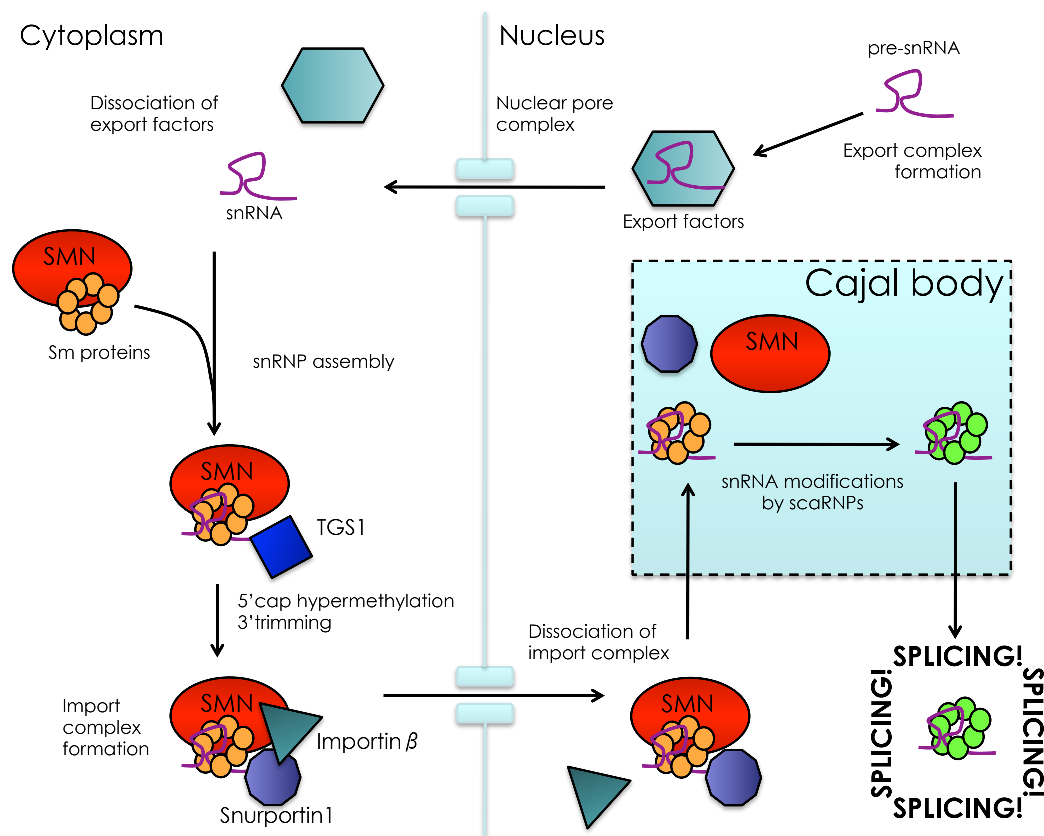

Figure 4: The life-cycle of U1, U2, U4 and *U5 snRNPs. The drawing shows the key steps of snRNP biogenesis. The matured snRNA is shown as by green Sm proteins. The matured snRNP complex either participates directly in splicing or is stored in nuclear speckles.*

2.7.4 snRNP Biogenesis

SnRNAs are a small group of non-polyadenylated ncRNAs that are highly abundant in the cell. They are classified either as Sm- or Lsm-class of RNAs depending on the structural motifs they contain and the type of proteins they associate with. U1, U2, U4 and U5 of the major spliceosome belong to the Sm-class and are characterized by a 7-methylguanosine cap at their 5'end (5'm7G), a 3'stem loop and an Sm-protein binding site. The U6 snRNA, on the other hand, belongs to the Lsm-class, which also forms a RNP but with Lsm-proteins and has a different type of 5'end and 3'stem loop[163,164].

The biogenesis of snRNPs prior to their assembly into the spliceosome is a complex process that includes both nuclear and cytoplasmic phases (Figure 4). All spliceosomal snRNAs except U6 are transcribed by a specialized form of RNA polymerase II[165], and then exported to the cytoplasm by an export complex consisting of the snRNA-specific adaptor protein PHAX, the export receptor chromosome region maintenance-1 (CRM1), the cap-binding complex and Ran GTPase[166,167].

Once out in the cytoplasm, the export complex dissociates as the SMN complex binds to the snRNAs[166,168]. The SMN complex, which will be described more in detail later, is a multimeric complex that recognises specific sequence elements in the snRNAs and brings together the snRNAs with a set of seven Sm-proteins to form the Sm-core RNP[169,170]. The order of interaction seems to be important for the assembly of functional snRNP. The SMN complex must first bind to Sm-proteins before binding to the snRNAs [171]. Although Sm-proteins are able to spontaneously assemble on snRNA *in vitro*[172,173], the SMN complex is required *in vivo* to facilitate the process and to ensure the specific binding of Sm-proteins to the correct RNA targets[171,174].

Upon assembly of the snRNP, SMN recruits TGS1 for the hypermethylation of the 5'cap to form a 2,2,7-trimethylguanosine (TMG) cap structure[175,176]. In addition, the 3'end is trimmed by a yet-to-be-identified exonuclease[177,178], which further facilitates the nuclear import. The formation of the TMG-cap triggers the assembly of the import complex. The nuclear import utilises the nuclear import receptor importinβ either by TMG cap-dependent or Sm-dependent pathway. The TMG cap-dependent pathway operates via the import adaptor snurportin-1, which binds the modified 5'cap of the snRNA and brings the snRNP complex together with importinβ[179] to allow for the re-import of the complex to the nucleus. In the Sm-dependent pathway, SMN has been shown to link the snRNP complex to importinβ and the interaction between SMN and importinβ has been demonstrated to be enough for the nuclear import of the snRNP complex[180,181]. However, the existence of an additional adaptor protein that mediates the interaction between the Sm/SMN protein and importinβ has been suggested [182]. Upon re-entry of the snRNP complexes into the nucleus via nuclear pore complexes[183], importinβ dissociates[184] and the complex localises to the Cajal bodies.

Within the Cajal body, scaRNPs perform site-specific pseudouridylation and 2'-*O*-

methylation of target snRNA residues[125], modifications that are essential for the function of the snRNPs[185]. The matured snRNP complex is either directed to perichromatin fibrils to participate in splicing or stored in nuclear speckles (interchomatin granule clusters) for later use[186]. There are also reports that suggest a transient stay of the mature snRNPs in the nucleolus prior to their localization in speckles[140]. Cajal bodies have also been implicated as sites for the formation of the tri-snRNPs, which are the central units of the spliceosome. The tri-snRNPs consists of U4/U6 and U5 snRNPs and are first assembled as a U4/U6 di-snRNP and then into a U4/U6.U5 tri-snRNP prior to entering the spliceosome. After splicing, the spliceosome disintegrates and individual U4, U6 and U5 snRNPs reassemble into a new tri-snRNP, before re-entering the next splicing cycle[126,187,188].

Unlike U1, U2, U4 and U5 snRNPs, U6 snRNP biogenesis is entirely nuclear. U6 is transcribed by RNA polymerase III, and in a series of steps, which is not fully understood, a heptameric ring of Lsm-proteins are added[189]. Also, unlike the other snRNPs, U6 snRNP undergoes maturation in the nucleolus by snoRNPs[190,191]. The mature form of U6 snRNP then localizes to the Cajal bodies for the formation of the U4/U6 di- and U4/U6.U5 tri-snRNPs prior to localizing to the nuclear speckles[126,187,188].

The SMN complex plays an essential role in snRNP assembly as well as in the import of the complex into the nucleus. Due to its roles in snRNP biogenesis it is also important for the Cajal body formation and function.

2.7.5 The Survival of Motor Neuron Complex

The SMN complex is a multiprotein complex that contains SMN, Gemin2-8 and Unrip. In addition to its function in the assembly of the spliceosomal snRNPs, it has also been suggested to have roles in the biogenesis of telomerase[192], microRNPs[193] and snoRNPs[194,195,196]. The SMN complex has been the focus of intense research due to its link to the neurodegenerative disease Spinal Muscular Atrophy (SMA), which is associated with loss or mutations of the *SMN1* gene[197,198]. The SMN complex is present both in the cytoplasm and in the nucleus, where it is highly enriched in nuclear structures called gems. Gems resemble Cajal bodies in number and size and colocalize with Cajal bodies in most adult tissue cell lines. However, in fetal tissues and certain types of cell lines, gems and Cajal bodies are separate distinct nuclear structures. Gems contain SMN and associated proteins but lack snRNPs and coilin. Their biological significance is not known[169].

SMN is the central component of the SMN complex. It has an RNA binding domain at its N-terminus[199], followed by a tudor domain that binds to arginine and glycine (RG) rich motifs, found in numerous proteins such as coilin and Sm-proteins[151,200,201]. These motifs are important for bringing the Sm-proteins together with snRNAs. In its C-terminal region, SMN has a proline rich region that acts as a protein binding domain[202] and a tyrosine and glycine rich region via which it oligomerizes. This self-association is important for the stability of the protein[203,204].

Although the precise function and stoichiometry of the individual components of the SMN complex is still not fully understood, it has been shown that Gemin2, 3, 5, 7 and 8 directly interact with SMN[169,205,206]. Gemin4 interacts with the complex via Gemin3, whereas Gemin6 interact via Gemin7[207,208]. The Unrip protein associates with the SMN complex through Gemin6 and 7[209]. Gemin2 is believed to play an essential role in the SMN complex by stabilizing it[210]. In fact, just a minimal complex consisting of SMN and Gemin2 is sufficient to facilitate Sm core formation[211,212], which suggests that there are redundant functions between the different components of the SMN complex in Sm-core assembly. Gemin3 is an RNA helicase that, together with Gemin4, is required for chaperoning of the snRNPs [207,213]. Gemin5 has been shown to directly bind snRNAs and is believed to be the specificity factor that allows the SMN complex to distinguish snRNAs from other cellular RNAs [214]. Gemin6, Gemin7 and Unrip form a complex required for efficient assembly of snRNPs and that associate with SMN via Gemin8[206].

2.7.6 Spinal Muscular Atrophy

SMA is the leading genetic cause of infant mortality worldwide, affecting one in 6000 infants[215]. It is a neuromuscular disease that causes the specific degeneration of motor neurons in the spinal cord. SMA is caused by reduced levels of the SMN protein. In humans, two genes, *SMN1* and *SMN2*, code for SMN and in 95% of SMA cases the *SMN1* gene is absent [198,216]. The clinical severity of the disease is linked to the copy number of *SMN2*. The *SMN2* gene is nearly identical to *SMN1*, but a C-T change in exon 7 leads to the frequent skipping of exon 7[217,218]. Thus, 90% of the *SMN2* transcripts encode a truncated unstable version of the SMN protein termed SMNΔexon7. However, the remaining 10% that encode a functional full-length protein is enough to compensate for the loss of the *SMN1* gene and allow the survival of some patients that have deletion of *SMN1*. Thus, increased copy number of SMN2 compensates for the loss of *SMN1* and leads to milder clinical symptoms. Patients with the most severe form of SMA (type I) have one or two copies of *SMN2,* patients with the less severe type II form of SMA have three or four copies, and the patients with the least severe form (type III) have 5 or 6 copies of *SMN2*[219,220].

The reason for the specific degeneration of motor neurons in SMA patients is still not well understood. Two hypotheses have been proposed to explain SMA. The first model is based on the role of the SMN complex in snRNP biogenesis and the second model is based on its function in neuronal mRNA transport[197,198].

In line with the first hypothesis, cells expressing reduced levels of SMN or SMN mutants are defective in snRNP assembly[221,222]. Moreover, expression profiling of tissues from SMA mouse model show tissue–specific alterations in pre-mRNA splicing and changes in repertoires of snRNPs [223]. This suggests that loss of SMN may give tissue-specific changes that might be specifically deleterious for motor neurons. However, the target genes that are specifically affected are still unknown. Moreover, SMN and snRNPs fail to localise to Cajal bodies in SMN mutant harbouring cells[182,224,225], a phenotype that is linked to SMA pathogenesis[225].

SMN has also been proposed to function in the intracellular trafficking of mRNAs in motor neurons[226,227,228]. It has been shown to be important for the localization of β-actin mRNA to the growth cones of motor neurons[228], which are the tips of the axons and the dendrites of the nerve cell. These actin-based extensions are important for the neuronal outgrowth, and rely on the constant synthesis of β-actin. In SMA transgenic mice, the lengths of dendrites are reduced while the numbers of motor neurons remain the same[228]. This, together with the fact that knockdown studies in zebrafish show deficit in motor neuron axonal outgrowth[229], suggest that dysfunction of neuronal mRNA transport may be the cause of SMA.

In addition to snRNP biogenesis and recycling, Cajal bodies have also been implicated in snoRNP maturation.

2.7.7 snoRNAs

SnoRNAs represent one of the most diverse groups of ncRNAs and more than 200 unique snoRNAs have been identified up-to-date [230,231]. Their main function is to guide site-specific modification of other ncRNAs, mainly ribosomal (r)RNAs , transfer (t)RNAs and snRNAs. However, they have also been implicated in pre-rRNA endonucleolytic processing as well as in targeting other RNAs than rRNA such as brain-specific mRNA in mammals [164,232]. They are divided either into C/D and H/ACA snoRNAs based on their sequence elements and secondary structures. While C/D snoRNAs are in general responsible for methylation, H/ACA snoRNAs guide the conversion of uridine to pseudouridine in a process called pseudouridylation. Depending on the type of snoRNA, they associate with a set of four core proteins, forming a snoRNP. Each snoRNA has a stretch of 10-20 nucleotides of complementary sequence to the target pre-RNA molecule, providing specificity to snoRNP[164,232].

Cajal bodies have also their own set of ncRNAs called scaRNAs, found exclusively in Cajal bodies.

2.7.8 scaRNAs

scaRNAs are related to snoRNAs but localize specifically to Cajal bodies and guide the modification/maturation of the snRNAs. ScaRNPs are in general more complex than snoRNP because they often contain two snoRNP domains. They can have either a C/D and a H/ACA domain, two box C/D or two box H/ACA domains. In addition, scaRNAs contain a conserved four-base sequence called a Cajal body (CAB) box that is essential for Cajal body localization. Specific CAB box-binding proteins bind this sequence and direct the scaRNAs to and/or retain scaRNAs in Cajal bodies. Mutations within the CAB box of a scaRNA mislocalizes it to the nucleolus[233,234].

2.7.9 The Telomerase Complex

A famous member of the scaRNA family is TERC, the telomerase RNA component. TERC, together with the telomerase reverse transcriptase (TERT), dyskerin and the newly discovered WRAP53 (alias TCAB1 and Wdr79) protein form the

telomerase holoenzyme that is required for maintaining the telomeres[235,236]. Telomeres are regions of repetitive DNA that protect the ends of chromosomes from deterioration. As cells divide the telomeres grow shorter until they reach a critical level that will induce senescence and eventually cell death. By maintaining telomeres, telomerase supports proliferative potential of stem/progenitor cells as well as cancer cells. In fact, the maintenance of the telomerase, either by overexpression of telomerase or by the alternative lengthening of telomeres (ALT) pathway, is considered to be one of the hallmarks of tumourigenesis[237,238,239]. Cajal bodies are important for telomere maintenance[129,235,240] and have been suggested to be involved in directing the telomerase complex to telomeres for extension[128,130,241].

The reason why our group got interested in the WRAP53 gene, at the first, was its close proximity to the p53 gene. Farnebo hypothesized that there may be an overlap between these two genes and that they might exert regulatory effects on each other. Indeed, after careful investigation she found that WRAP53 does overlap p53 and this discovery was the start of long journey that has taken us from regulatory antisense transcripts to nuclear architecture and cancer.

2.8 WRAP53 – IN ITS INFANCY

The *WRAP53* gene has three starting exons: 1α, 1β and 1γ with seemingly different promoters (Figure 5A). Transcripts initiated from these exons are referred to as WRAP53α, WRAP53β and WRAP53γ. *WRAP53* is a heavily alternatively spliced gene that gives rise to at least 17 different transcripts, several of which code for the WRAP53 protein (also called TCAB1 and Wdr79). Exon 1α of *WRAP53* overlaps *p53* exon 1 in opposite direction and transcripts starting from this exon regulate p53 levels (further described in Paper I). WRAP53β transcripts are the most abundant variant followed by WRAP53α transcripts, while the least prevalent transcripts originate from exon 1γ. Considering the low abundance of WRAP53α and WRAP53γ transcripts, and that knockdown of WRAP53α and WRAP53γ does not affect WRAP53 protein levels, it is highly likely that WRAP53 proteins originate from WRAP53β transcripts. Nonetheless, WRAP53α and γ transcripts also seem to have the capacity to give rise to the full-length protein[11].

The WRAP53 protein contains a proline-rich region in the N-terminus, followed by a WD40 repeat domain and a glycine-rich region in the C-terminus (Figure 5B). WD40 motifs are involved in protein-protein as well as in protein-RNA interactions[242]. They are believed to function as platforms to bring several proteins together and to allow the formation of macromolecules. It is a common structural domain that exists in a variety of proteins involved in everything from apoptosis and cell cycle control to RNA processing and protein degradation.

In the last few years there has been a major boom in the WRAP53 field. From being completely unknown, WRAP53 has now been identified as a regulator of p53 (Paper I)[11] and as a major player in Cajal body formation and functions (Paper II)[234,235,243,244]. Around the same time as our paper describing the function of the antisense transcript was published, two papers came out

revealing a role of the WRAP53 protein in directing scaRNAs, including TERT, to Cajal bodies[234,235].

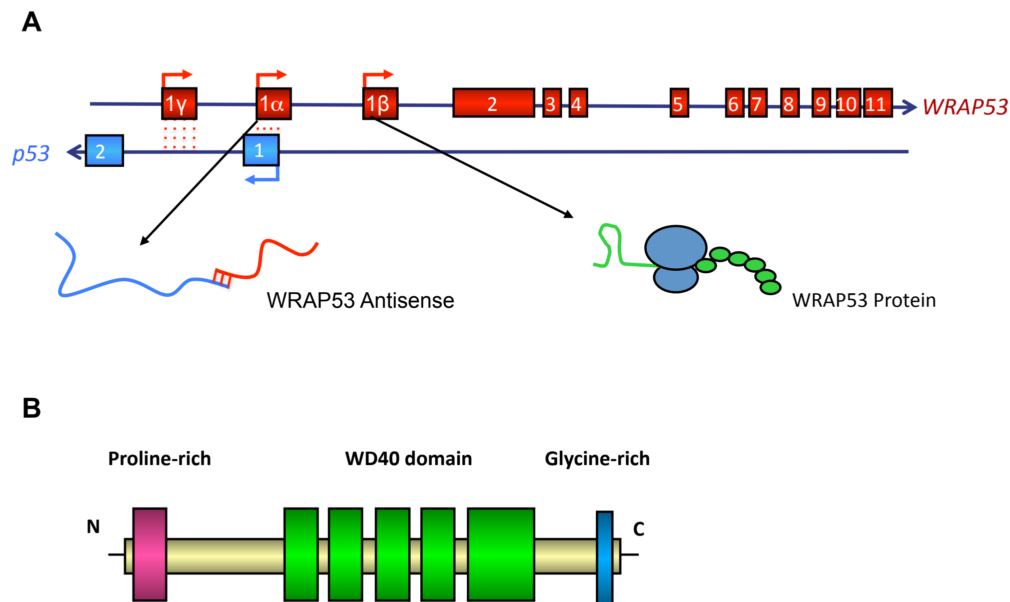

*Figure 5: Schematic illustration of the WRAP53 gene locus (**A**) and the WRAP53 protein (**B**).*

Venteicher and colleagues identified WRAP53 as a component of the telomerase complex. They showed that WRAP53 binds to TERC and that WRAP53 is important for directing the complex to Cajal bodies. Interestingly, they showed that WRAP53 knockout cells exhibited shorter telomeres compared to control cells[235]. This initial discovery of WRAP53 in the transport of the telomerase complex to Cajal bodies was followed by the finding that WRAP53 acts as a general transport mediator of scaRNAs to Cajal bodies. WRAP53 was found to bind the CAB domain of scaRNAs and to direct them to Cajal bodies[234]. Our recent finding that WRAP53 also directs the SMN complex to Cajal bodies (Paper II) further supports the role of WRAP53 as a specific delivery system of the Cajal bodies. It also emphasizes the importance of WRAP53 in the formation and integrity of these nuclear structures[243], a finding that has further been verified in *Drosphila*, where a mutant lacking WRAP53 was also found to lack Cajal bodies[244].

The *WRAP53* gene has also been implicated in human cancers. Two single nucleotide polymorphisms (SNPs) have been associated with increased risk for breast cancer[245] and aggressive ovarian cancer[246]. One of the SNPs lies with the coding region of *WRAP53* and results in the amino acid change R68G, which suggests that alterations of the WRAP53 protein could contribute to cancer. Furthermore, gene expression profiling revealed increased levels of WRAP53 in sporadic primary hyperparathyroidism compared to normal parathyroid tissue[246]. These data are in line with our discovery that WRAP53 may have oncogenic properties and seems to be essential for cancer cell survival (Paper III)[247].

3. AIMS OF THIS THESIS

The overall purpose of this thesis was to characterize the function of the *WRAP53* gene and its link to cancer.

Specific aims of the papers:

I. To elucidate the role of the WRAP53 antisense transcript in the regulation of p53.

II. To investigate the function of the WRAP53 protein in Cajal body formation and function.

III. To assess the potential of the WRAP53 protein as a target for cancer therapy.

4. RESULTS AND DISCUSSION

4.1 PAPER I

This study was initiated because of the genomic localization of *WRAP53* on chromosome 17. Considering the close proximity of the *WRAP53* gene to the *p53* gene, we hypothesised that these two genes might overlap. Careful investigation revealed that *WRAP53* has three starting exons, 1α, 1β and 1γ, and that exon 1α overlaps exon 1 of *p53* in an opposite direction. This finding suggested the existence of a *cis*-acting regulatory circuit between the two overlapping transcripts. In support of this idea, expression profiling performed in cancer cell lines showed a positive correlation between the WRAP53α and p53 transcripts.

Initial knockdown experiments showed that silencing of WRAP53 downregulated p53 RNA and protein levels. Conversely, overexpression of WRAP53α increased p53 levels. However, this regulation occurred in a non-reciprocal manner since alterations of p53 levels had no effect on WRAP53 expression. Several lines of evidence clearly pinpointed the antisense RNA as the regulator of p53 expression and not the non-overlapping transcripts nor the protein. First, overexpression of only the constructs that contain the overlapping exon 1α region induced p53 levels. In fact, overexpression of just the overlapping region that does not encode any protein was enough to increase p53 expression. Second, although the majority of the transcripts giving rise to the protein are initiated from exon 1β, only siRNAs targeting exon1α transcripts downregulated p53 expression. Third, p53 levels could be rescued upon WRAP53 knockdown by expression of WRAP53α cDNA constructs containing the overlapping region but not expressing WRAP53 protein due to a premature stop codon. These results demonstrated convincingly that p53 regulation is indeed mediated by the WRAP53α transcript via the overlapping region.

Next we went on to characterize the molecular mechanism underlying WRAP53α-mediated regulation of p53 expression. Our data pointed to a predominant post-transcriptional regulation. Since silencing of WRAP53 only affected p53 mature mRNA and not p53 pre-mRNA, and the fact that the regulation seems to occur both in the nucleus and in the cytoplasm, more or less excluded regulation at a transcriptional level. In support of these data, *in vitro* experiments where the p53 promoter was put in front of luciferase showed no changes in luciferase activity upon altered levels of WRAP53α. Importantly, experiments using reporter constructs where exon 1 of p53 was fused to luciferase cDNA, showed a dramatic decrease in luciferase activity upon WRAP53 depletion. These data support a post-transcriptional regulation mediated through the 5'UTR of p53.

In line with these data, transient transfection of 2'-*O*-methyl oligonucleotides targeting either exon1α of WRAP53 or exon1 of p53 resulted in decreased levels of p53. 2'-*O*-methyl oligonucleotides are commonly used to the block binding of microRNA to their targets without the concomitant degradation of the microRNA. The fact that treatment with these oligos reduced p53 levels suggests that WRAP53-mediated regulation of p53 occurs via WRAP53α/p53 RNA

interaction. Since transcription of WRAP53α gives rise to a perfectly matching complementary RNA to p53 mRNA, it would be feasible that WRAP53α forms RNA duplexes with p53 mRNA. However, in spite of our extensive attempts to find RNA duplexes in living cells, we were unable to detect them *in vivo*.

While our inability to detect WRAP53α/p53 duplexes may be due to experimental limitations, it could also reflect the transient and labile nature of the interaction. A transient binding would be enough to change the secondary structure of the p53 mRNA, leading to a more stable conformation. This way, one WRAP53α transcript would be able to target several p53 transcripts, which could also explain how WRAP53α is capable of protecting p53 mRNA in excess. Also, the transient nature of the interaction may be essential in order to avoid activation of the cellular interferon-mediated pathway that is triggered by the presence of viral RNA duplexes, resulting in shutdown of protein synthesis and culminating in apoptosis. Another possibility is that the interaction does not rely on base-pairing interactions and is instead via the formation of secondary and tertiary structures. WRAP53α might also mimic regulatory elements within the p53 exon1 and block the binding of destabilizing factors to p53 mRNA. Furthermore, the recent finding that the 3'UTR of p53 mRNA folds back on itself and interacts with the 5'UTR raises the possibilities that WRAP53α might also affect regulatory sequences in the 3'UTR.

Although the molecular mechanism underlying WRAP53α-mediated regulation of p53 remains to be elucidated, the biological importance of this regulatory pathway is clear; WRAP53α is essential for p53 DNA damage response. This is supported by the fact that depletion of WRAP53α prevents the induction of p53 and several of its target genes, including p21 and PUMA, upon DNA damage. Furthermore, ectopic expression of WRAP53α potentiates p53-mediated apoptosis. Interestingly, we also observed an increase of both p53 and WRAP53α transcripts upon DNA damage. This suggests that WRAP53α is not only important for maintaining steady-state levels of p53 but is also induced upon stress to confer greater stability and sustain increased levels of p53. Altogether, these results reveal an important role of WRAP53α in p53 DNA damage response.

The identification of WRAP53 adds another layer of complexity to the regulation of p53. Although additional regulatory mechanisms to an already heavily controlled gene such as p53 may seem redundant, it reflects the importance of maintaining the appropriate levels of p53 at all times. Since WRAP53α is induced upon DNA damage, we can assume that it has a role in maintaining p53 levels in response to DNA damage. Although p53 is initially stabilized at a post-translational level, an increase in WRAP53α ensures sustained *de novo* protein synthesis for enhanced and prolonged p53 response. Considering the oscillating expression pattern of p53 upon DNA damage[57], the induction of WRAP53α could potentially allow for an increased p53 induction after the initial activation to shift from cell cycle arrest to apoptosis.

WRAP53α is, in addition, important for retaining basal p53 levels, suggesting a role in p53 functions that are not stress-induced. The important role of WRAP53α in p53 action also opens the possibility that dysfunction of WRAP53α

itself may contribute to cancer. Future studies will reveal the exact molecular pathway of WRAP53α-mediated p53 regulation and will also shed light on the importance of WRAP53α in different aspects of p53 function.

4.2 PAPER II

This paper was initiated by the observation that the WRAP53 protein localizes to Cajal bodies and that WRAP53 depletion leads to disruption of these nuclear organelles. Around the same time as our initial observations, two papers came out identifying WRAP53 as a Cajal body specific protein, important for the localization of scaRNAs to these structures[234,235].

Functional studies altering WRAP53 levels revealed the importance of WRAP53 in Cajal body formation and integrity. Knockdown of WRAP53 using two different siRNA oligos led to the disappearance of specifically Cajal bodies with a concomitant mislocalization of Cajal body components, including coilin, SMN, Sm, Gemin2 and Gemin3. We also obtained similar results in cells overexpressing FLAG/GFP tagged-WRAP53 at high levels. While cells with low ectopic expression of WRAP53 displayed a similar distribution pattern as endogenous WRAP53, exogenous expression of WRAP53 at high levels led to disruption of Cajal body structure and the subsequent mislocalization of its components. Neither silencing nor overexpression of WRAP53 altered the expression levels of Cajal body components, suggesting a direct role of WRAP53 in Cajal body maintenance. This idea was in line with previous studies identifying a role for WRAP53 in directing scaRNPs to Cajal bodies[234,235].

In contrast to previous studies, immunofluorescence (IF) analyses in our lab showed a different intracellular distribution pattern of WRAP53. We detected a clear cytoplasmic staining in addition to the described Cajal body localization. Comparing the cellular distribution pattern of WRAP53 to other known Cajal body components, we saw a strong resemblance to the localization of the SMN complex. Thus we hypothesised that WRAP53 might be interacting with this complex, perhaps directing it to Cajal bodies. Indeed, IF, immunoprecipitation (IP) and *in situ* proximity ligation assay (PLA) experiments demonstrated that WRAP53 both co-localizes and interacts with components of the SMN complex both in the nucleus and in the cytoplasm. In addition, WRAP53 IPs in coilin- or SMN-depleted cells revealed that WRAP53 interacts with SMN independently of coilin, and with coilin independently of SMN. Strikingly, IPs using coilin or SMN antibodies in WRAP53 depleted cells revealed that WRAP53 is required for the interaction between these two proteins. Since SMN and coilin have previously been shown to interact directly[151], these data suggested that WRAP53 brings these proteins together to allow them to bind each other. Thus, WRAP53 appears to have a role in directing the SMN complex to Cajal bodies.

Considering that WRAP53 interacts with the SMN complex in the cytoplasm and in the nucleus, we hypothesized that WRAP53 may have an additional role in the nuclear import of the complex. As previously mentioned, the SMN complex together with the snRNP utilises the importinβ pathway to enter the nucleus. This is achieved either by the TMG cap-dependent or the Sm-dependent pathway [179,180]. Interestingly, the existence of an additional adaptor protein has been

suggested, which mediates the interaction between importinβ and SMN in the Sm-dependent pathway[182]. Indeed, our results showed that WRAP53 facilitates the nuclear import of the SMN complex. Depletion of WRAP53 led to increased levels of SMN in the cytoplasm with a subsequent decrease in the nucleus. Moreover, the interaction between SMN and importinβ was disrupted in cells depleted for WRAP53. Thus, WRAP53 seems to bind to the SMN complex in the cytoplasm, facilitate its nuclear import via importinβ and direct it to Cajal bodies.

WRAP53 may also be involved in the pathogenesis of the neurodegenerative disease SMA. We found that the interactions between SMN and WRAP53 were disrupted in fibroblasts from SMA patients and that this correlated with reduced number of SMN foci in the nuclei of these cells. As reduced nuclear foci of SMN correlates with the severity of the disease, an inability of mutant SMN to bind WRAP53 could contribute to SMN dysfunction in SMA.

Our results also suggest that WRAP53 has functions in the Cajal body beyond its role in snRNP biogenesis. Cajal bodies have been suggested to have compartments constituting the different activities[140]. The fact that WRAP53 but not SMN knockdown removes both canonical and residual Cajal bodies indicates that WRAP53 is important for the additional ongoing processes in these structures.

From this study, we conclude that WRAP53 is essential for Cajal body formation and maintenance. WRAP53 facilitates the nuclear import of the SMN complex and further mediates the interaction between the SMN complex and coilin. Our findings, together with what has previously been published, establish a crucial role of WRAP53 in Cajal body formation and function.

4.3 PAPER III

During our functional studies of the WRAP53 protein, we observed that WRAP53 depletion resulted in massive cell death. Time-lapse experiments revealed that starting from approximately 48h post siWRAP53 transfection, the cells begin to die and that by 96h most cells are dead. In depth characterization of the cell death revealed that cells undergo classical mitochondrial-dependent apoptosis. This conclusion was supported by the fact that we observed morphological changes in WRAP53-depleted cells associated with apoptosis, such as membrane blebbing and cell shrinkage. In addition, we detected caspase activation and PARP cleavage, two molecular processes that are strongly linked to apoptosis. Moreover, we observed cyt c release and a drop in mitochondrial membrane potential, indicating a disruption of the mitochondria upon WRAP53 depletion. The effect on the mitochondria seems to be linked to Bax and Bak activation since we observed activation of Bax and Bak already at 48h. However, the strongest evidence supporting a mitochondrial-dependent apoptosis was the fact that we could rescue the observed cell death by ectopic expression of Bcl-2.

Most of the initial studies to characterize the apoptotic pathway were performed in U2OS and HeLa cells. Next we extended our phenotypic studies to other cell

types, including additional human cancer cell lines as well as primary cells. We observed similar effects upon WRAP53 depletion in H1299 and HEK293 cancer cells; however, knocking down WRAP53 in three different primary cells, namely HDF, AG68014 and MCF10a, did not result in any significant cell death. Thus, cancer cells seems to be more sensitive to WRAP53 depletion than primary cells, identifying WRAP53 as a target for cancer therapy.

The fact that cancer cells appear to have acquired a dependency on WRAP53 expression encouraged us to investigate whether WRAP53 has oncogenic properties. In line with this idea, we found that WRAP53 levels are in general elevated in cancer cells compared to primary cells and, more importantly, anchorage-independent transformation assays using murine NIH3T3 cells displayed increased numbers of colonies in WRAP53 overexpressing cells compared to control cells. These data support a role of WRAP53 in tumourigenesis by promoting cell survival.

Next we looked at the impact of WRAP53 expression in primary tumours. Interestingly, WRAP53 levels were found to be correlated to patient prognosis and intrinsic radiosensitivity, a measurement of the sensitivity of the cancer cells to radiotherapy, in head and neck squamous cell carcinoma cell lines derived form primary tumours. We found that enhanced WRAP53 expression was correlated to poor outcome and low intrinsic radiosenstivity, which supports a role of WRAP53 in the survival of cancer cells *in vivo* and indicates that WRAP53 may be a novel marker for the prognosis of head and neck cancer.

The question of how the WRAP53 protein promotes cell survival and transforms cells remains to be elucidated. However, these actions of the WRAP53 protein seem to be independent of WRAP53α-mediated regulation of p53. The reason is that the protein is mainly transcribed from the WRAP53β transcript, which is not involved in the p53 regulation, and also because knockdown of WRAP53 induces cell death in p53 wt and null cells. In fact, no currently known functions of the WRAP53 protein can account for the cell survival promoting ability of WRAP53 in a satisfactory way. The role of WRAP53 in telomere extension could partially explain the oncogenic properties of WRAP53 as well as the cancer-cell dependency. However, ectopic expression of WRAP53 transforms cells with already high telomerase activity and depletion of WRAP53 kills telomerase null cells that rely on the ALT pathway, suggesting telomerase-independent functions of WRAP53.

Furthermore, no solid data links Cajal bodies to tumour development and progression and Cajal bodies are not essential for cell survival. Coilin has recently been assigned functions in maintaining genomic stability, independent of its function in Cajal bodies. However, knockdown of coilin does not kill cancer cells (data not shown). In contrast, reduced levels of SMN have been reported to induce mitochondrial associated cell death. Nonetheless, since cells survive in absence of Cajal bodies, the function of WRAP53 in directing the SMN complex to Cajal bodies should not be the underlying mechanism. Also, SMN has not previously been implicated in cancer and no enhanced levels of SMN were observed in cancer cells compared to primary cells. Altogether, these results suggest additional functions of WRAP53 in cancer cell survival.

From this study, we conclude that WRAP53 expression is elevated in cancer cells compared to primary cells and that WRAP53 has oncogenic properties. Furthermore, the fact that cancer cells are more sensitive to depletion of WRAP53 than primary cells opens up possibilities for therapeutic intervention. Overall, our findings establish a clear link between the WRAP53 protein and cancer and identify WRAP53 as a target for cancer therapeutics and as a prognostic marker.

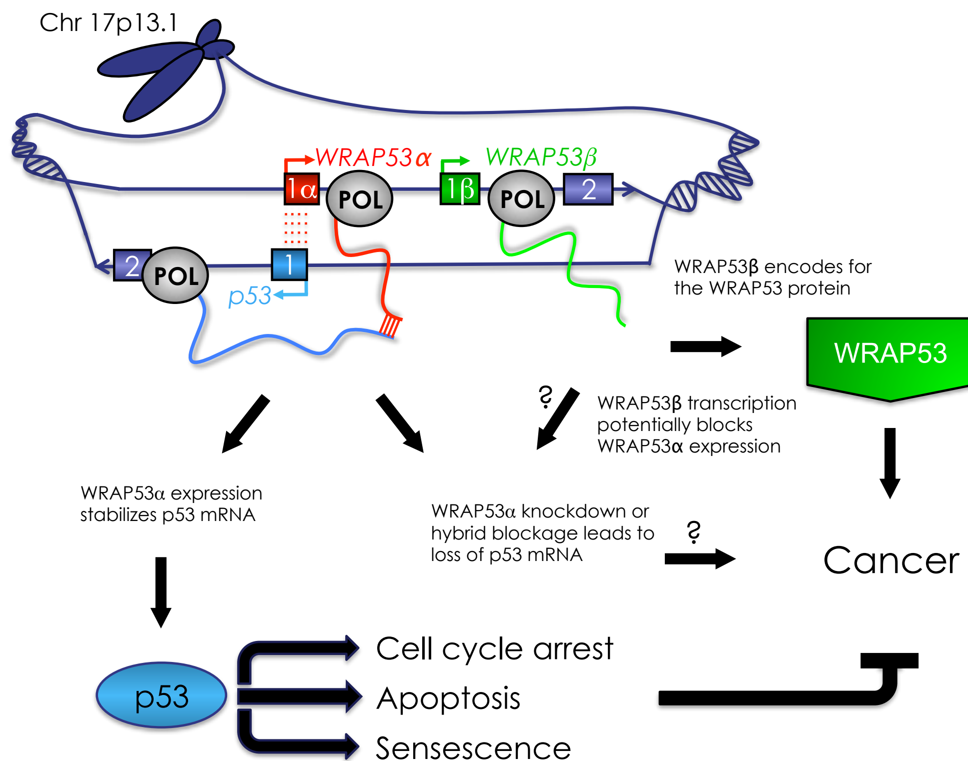

Figure 6: Schematic illustration of the WRAP53 gene locus and the potential implications of expressing the different WRAP53 transcripts.

4.4 THE TWO FACES OF *WRAP53*

WRAP53 is a gene of multiple functions; it gives rise to an antisense transcript that regulates the actions of the tumour suppressor p53 and it encodes for a protein with oncogenic properties (Figure 6). These somewhat contradictory functions of *WRAP53* are possible because of its genomic arrangement. WRAP53 has three starting exons and, depending on the origin of the transcript, it can have different activities. Transcripts originating from exon 1α stabilize p53 and transcripts mainly starting from exon 1β code for the protein. Since WRAP53α and WRAP53β seem to have separate promoters, they allow for independent regulation of each transcript. Moreover, the induction of WRAP53α seems to repress the expression of WRAP53β, suggesting inter-regulation between the transcripts[11]. Thus, the upregulation of the WRAP53β transcripts could have dual growth-promoting effects: by inducing the WRAP53 protein and simultaneously getting rid of p53 through blockage of WRAP53α expression. On

the contrary, the expression of WRAP53α could act on a double level to suppress growth by promoting p53 expression and preventing WRAP53 protein expression. This could be a way of nature to balance the growth promoting and growth suppressive actions of important cellular regulators and raises the possibility that other sense/antisense genes have acquired a similar genomic arrangement to allow for a similar type of regulation.

RNA plays an important role in WRAP53 functions; WRAP53α transcripts act as regulatory RNAs to stabilize p53 and the WRAP53 protein binds to scaRNAs. In fact, all the currently characterized functions of the WRAP53 protein revolve around RNA-protein complexes. WRAP53 protein was shown to be important for the localization of scaRNPs and the telomerase complex to Cajal bodies and we show that WRAP53 directs the SMN complex together with snRNPs to the same nuclear structure (Figure 7)[234,235,243]. Also, it was shown that WRAP53 binds directly to scaRNAs and the inability of the scaRNAs to bind WRAP53 leads to their mislocalization to nucleoli[234]. Furthermore, interaction between WRAP53 and the catalytic unit of the telomerase complex, TERC, was disrupted by ribonuclease A (RNase A) treatment[235], indicating that their association is mediated via RNA. Similar experiments performed in our lab showed that the WRAP53 interaction with SMN, importinβ and coilin is dependent on RNA (unpublished data). Thus, RNA seems to play an important role in the association of the WRAP53 protein with other proteins.

Although, previous studies described a specific binding of WRAP53 to the CAB domains of scaRNAs, our data showing that the interaction between WRAP53 and the SMN complex is mediated via RNA, suggests that WRAP53 binds to a broader repertoire of RNAs. This opens up the possibility that WRAP53 may be involved in the processing/transport of other types of RNAs. Considering the large number of orphan snoRNAs (snoRNAs with no known sequence complementarities), it is plausible that WRAP53 is part of these complexes. Also since the majority of WRAP53 resides in the cytoplasm, it may have functions in the biogenesis of other types of cytoplasmic RNAs, including mRNAs. The SMN complex has been shown to play a role in axonal RNA transport [226,227,228], and since WRAP53 binds to SMN in the cytoplasm, it is likely that it has a role in other SMN functions beside snRNP biogenesis.

Many questions remain to be answered regarding the function and the biological significance of the *WRAP53* gene. Nevertheless, the therapeutical potential is encouraging. Alteration of WRAP53α levels, such as upregulation of WRAP53α to enhance p53 expression or downregulation of WRAP53α to inhibit mutant p53 action, could turn out to be promising strategies for cancer treatment. Furthermore, considering that cancer cells are more sensitive to knockdown of the WRAP53 protein highlights the protein as a putative target for cancer therapeutics. Future studies will surely provide us with answers.

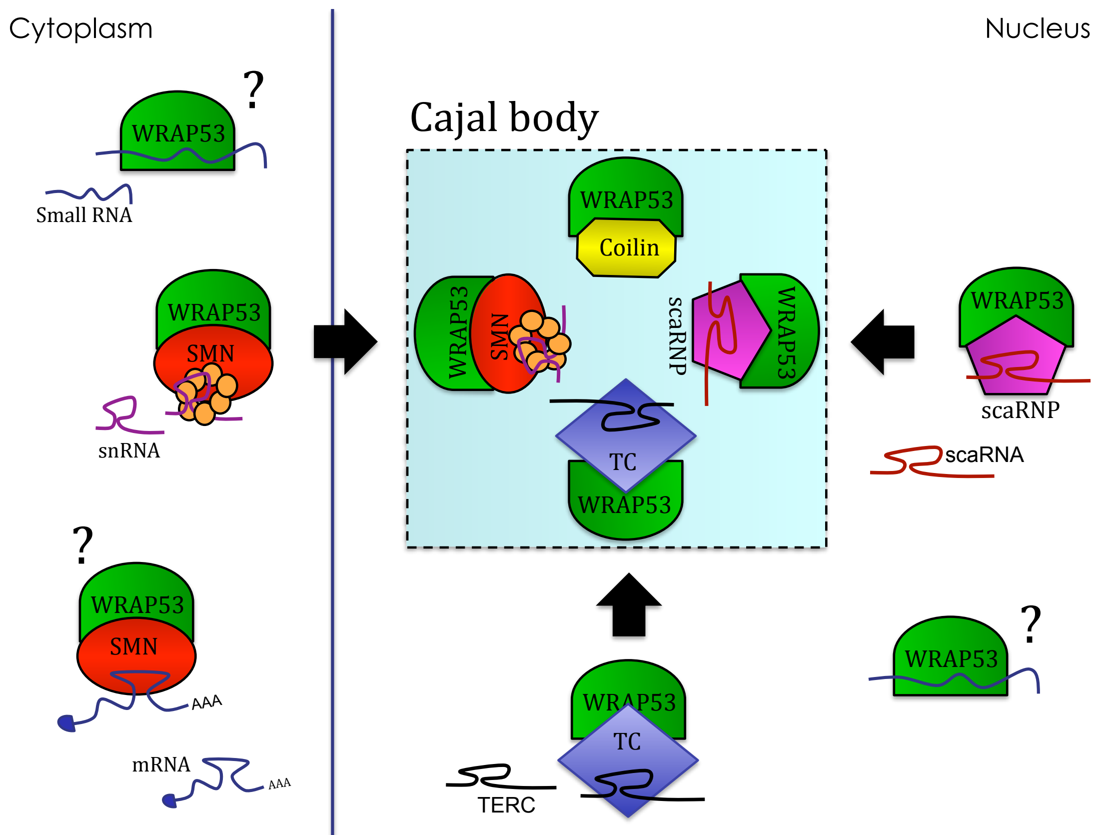

Figure 7: The various roles of the WRAP53 protein. WRAP53 act as a transport system for the Cajal bodies, bringing together the different components. WRAP53 may have additional functions in the cytoplasm together with the SMN complex. Moreover, it might be involved in the processing/transport of other types of RNAs in the nucleus or in the cytoplasm. The type of RNAs in the different complexes are illustrated next to each complex. TC stands for Telomerase complex

4.5 CONCLUSIONS IN SUMMARY

Paper I

WRAP53 is a natural antisense transcript of p53 that regulates the action of p53 upon DNA damage by stabilizing p53 mRNA.

Paper II

WRAP53 is essential for Cajal body formation and maintenance. It binds to the SMN complex in the cytoplasm and directs it to the Cajal bodies.

Paper III

WRAP53 is overexpressed in cancer and has oncogenic properties. Knockdown of WRAP53 leads to massive cell death of cancer cells but not of primary cells, identifying WRAP53 as a potential target for cancer therapy.

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Sofia, my first student! Since you started in the group, my life has definitely become much easier ☺ That is not only because of your brilliant research skills and "I know what you said last summer, word for word" type of memory which is like basically having a camcord along with you recording everything that has been said and done. It is also because you were the first one to understand my type of humour after 3 years here at CCK! You are the perfect partner in crime in joking with people, the wedding joke being already a classic.

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