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THE ROLE OF THE RNA-BINDING PROTEIN WIG-1 IN POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

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Karolinska Institutet

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The role of the RNA-binding protein Wig-1 in post-transcriptional regulation of gene expression

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To my family

ABSTRACT

The p53 transcription factor is activated by cellular stress. This triggers transcriptional activation of a number of p53 target genes, leading to responses such as cell cycle arrest and/or induction of apoptosis. Wig-1 is a p53 target gene and its RNA and protein levels increase after p53 protein activation. Wig-1 is a RNA-binding zinc finger protein with affinity to double-stranded RNA and it is involved in regulation of mRNA stability. In this thesis, I focused on the characterization of the Wig-1 protein function, on the identification of its bound RNA targets and on the elucidation of the biological implication of their regulation.

We found that Wig-1 belongs to the group of proteins known as AU-rich element binding proteins (ARE-BPs) and plays a role in regulation of post-transcriptional gene expression targeting mRNAs containing AU-rich elements (ARE) in their 3'UTRs. In paper I, we showed that Wig-1 stabilizes p53 mRNA by preventing its deadenylation and that this regulation is mediated through direct binding of Wig-1 to a U-rich element (a subgroup of AREs) in the 3'UTR of p53 mRNA. In paper II, we found that Wig-1 binds to N-Myc mRNA and positively regulates it through an ARE in the 3'UTR. We also showed that Wig-1 knockdown in neuroblastoma cells carrying amplified N-Myc leads to cell differentiation and repressed cell growth as a consequence of Wig-1 regulation of N-Myc RNA stability. In paper III we performed microarray gene expression analysis after Wig-1 knockdown in the colon cancer cell line HCT116 and found a large group of mRNAs that are directly or indirectly affected by Wig-1. We also discovered that Wig-1 knockdown is affecting cell cycle and the apoptotic response to stress through regulation of the p53 target genes FAS and 14-3-3σ. We could demonstrate that FAS mRNA regulation is dependent on Wig-1 binding to an ARE on FAS 3'UTR. At last, in paper IV, we performed RNA-immunoprecipitation followed by deep sequencing in order to identify genome-wide Wig-1 associated mRNAs. The analysis revealed that Wig-1 binds a large number of mRNAs most of which are functionally connected to the cell cycle pathway. Moreover, sequence analysis revealed that AREs are highly enriched in the 3'UTRs of these Wig-1-bound mRNAs.

In conclusion, this thesis provides a comprehensive view of the RNA-binding properties of Wig-1 and helps to better define the Wig-1-RNA interaction network. Our data establish Wig-1 as an AU-rich element binding protein involved in regulation of post-transcriptional gene expression of many mRNAs such as the p53 tumor suppressor and its transcriptional target FAS, the N-Myc oncogene and several other targets, ultimately affecting cell cycle progression and cell proliferation. Moreover, we provide additional insights into preferred Wig-1 RNA binding motifs. Additionally, as Wig-1 is a target of the p53 transcription factor, we gained further understanding of the p53-mediated tumor suppression through its target Wig-1, extending the frontiers of gene expression control from transcriptional to post-transcriptional level.

LIST OF SCIENTIFIC PAPERS

I. Anna Vilborg, Jacob A. Glahder, Margareta T. Wilhelm, **Cinzia Bersani**, Martin Corcoran, Salah Mahmoudi, Maiken Rosenstierne, Dan Grandér, Marianne Farnebo, Bodil Norrild, and Klas G. Wiman.
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. Anna Vilborg, **Cinzia Bersani**, Malin Wickström, Lova Segerström, Per Kogner, Klas G. Wiman.
Wig-1, a novel regulator of N-Myc mRNA and N-Myc-driven tumor growth.
Cell Death Dis. 2012 Apr 19;3:e298

III. **Cinzia Bersani**, Lidi Xu, Anna Vilborg, Weng-Onn Lui, Klas G. Wiman.
Wig-1 regulates cell cycle arrest and cell death through the p53 targets FAS and 14-3-3σ.
Oncogene. 2014 Aug 28;33(35):4407-17

IV. **Cinzia Bersani**, Mikael Huss, Stefania Giacomello, Lidi Xu, Anna Vilborg, Andrey Alexeyenko, Weng-Onn Lui and Klas G. Wiman
Genome-wide identification of Wig-1 mRNA targets by RIP-Seq analysis.
Manuscript

Related publications

Anna Vilborg, **Cinzia Bersani**, Margareta T. Wilhelm, Klas G. Wiman.
The p53 target Wig-1: a regulator of mRNA stability and stem cell fate?
Cell Death Differ. 2011 Sep;18(9):1434-40. Review.

CONTENTS

LIST OF ABBREVIATIONS

4-SU	4-thiouridine
5-Fu	5-fluorouracil
5'TOP	5′ terminal oligopyrimidine
6-SG	6-thioguanosine
ADAR	adenosine deaminase, RNA-specific
AGO	argonaute protein
AKT3	v-akt murine thymoma viral oncogene homolog 3
AluSx	Arthrobacter luteus element Sx
AluY	Arthrobacter luteus element Y
AMD	AU-rich element mediated decay
AMD1	adenosylmethionine decarboxylase 1
Apo1/CD95	apoptosis antigen 1/cluster of differentiation 95
APP	amyloid precursor protein
ARE	adenylate-uridylate rich element
ARE-BP	adenylate-uridylate rich elements binding protein
ARF	ADP ribosylation factor
ASD	autism spectrum disorder
ASO	antisense oligonucleotide
ASPP1	apoptosis-stimulating protein of p53, 1
ASPP2	apoptosis-stimulating protein of p53, 2
AUF1	AU-rich element RNA-binding protein 1
AUTS2	autism susceptibility candidate 2
Bax	BCL2-associated X protein
Bid	BH3 interacting domain death agonist
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
BRAC1	breast cancer 1, early onset
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRD-Box	bearded-box

BRD7	bromodomain containing 7
BRF-1/2	B -related factor 1/2
BRK	baby rat kidney
Brn3	brain-specific homeobox/POU domain protein 3
C2H2	cys2/his2-type zinc finger
CAV1	caveolin 1
CCNG1	cyclin G1
Ccr4-Not	chemokine (C-C motif) receptor 4 - negative regulator of transcription
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN2A	cyclin-dependent kinase inhibitor 2A
CHEK1	checkpoint kinase 1
ChIP	chromatin immunoprecipitation
CLIP	cross-linking and immunoprecipitation
CNOT6	CCR4-NOT transcription complex, subunit 6
CNS	central nervous system
Co-IP	co-immunoprecipitation
CpG	cytosine-phosphate-guanine
CRD	coding region determinant
DAP5	death-associated protein 5
Dcp1/Dcp2	decapping mRNA 1/ decapping mRNA 2
DD	death domain
DICER1	double-stranded RNA-specific endoribonuclease 1
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DYRK2	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
EIF4E	eukaryotic translation initiation factor 4E
ELF1	E74-like factor 1 (ets domain transcription factor)
ESC	embryonic stem cell
EWS	Ewing sarcoma RNA binding protein 1
FADD	FAS-associated death domain-containing protein

FAS	FAS cell surface death receptor
FASL	FAS cell surface death receptor ligand
GC	guanine-cytosine
GU-rich	guanylate-uridylate-rich
GUS	glucuronidase
H3K27me3	tri-methylation of lysine 27 on histone H3
H3K36me3	tri-methylation of lysine 36 on histone H3
H3K4me3	tri-methylation of lysine 4 on histone H3
hCAS/CSE1L	human cellular apoptosis susceptibility/ chromosome segregation 1-like protein
HIF1A	hypoxia inducible factor 1, alpha subunit
HIPK2	homeodomain interacting protein kinase 2
HITS	high-throughput sequencing
hnRNP D	heterogeneous nuclear ribonucleoprotein D
hnRNPA2/B1	heterogeneous nuclear ribonucleoprotein A2/B1
hnRNPC1/C2	heterogeneous nuclear ribonucleoprotein C1/C2
HuD	human antigen D
HuR	human antigen R
Hzf	hematopoietic zinc finger
iASSP	inhibitor of apoptosis stimulating protein of p53
iCLIP	individual-nucleotide resolution CLIP
IMMP2L	inner mitochondrial membrane peptidase 2 like
INFβ	interferon beta
IRE	iron responsive elements
IRES	internal ribosome entry site
JAZ	just another zinc finger protein
KRAS	Kirsten rat sarcoma viral oncogene homolog
KSRP	KH type-splicing regulatory protein
L-DOPA	levo- dihydroxyphenylalanine
LocARNA	local alignment of RNA
m^7G-cap	7-methylguanylate cap

MAD2L1	mitotic arrest deficient 2-like protein 1
MAX	MYC associated factor X
MDM2	mouse double minute 2 human homolog
MDM4	mouse double minute 4 human homolog
miRNA	microRNA
mRNA	messenger RNA
MTHFD2	methylenetetrahydrofolate dehydrogenase 2
MYST	MOZ, YBF2/SAS3, SAS2 and TIP60 protein 1
N-Myc	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NGF	nerve growth factor
NHGRI	national human genome research institute
NPY	neuropeptide Y
NRF1	nuclear respiratory factor 1
ORF	open reading frame
p53AIP1	tumor protein p53 regulated apoptosis inducing protein 1
PAG608	p53-activated gene 608
Pan2/Pan3	PABP1 dependent poly A specific ribonuclease subunit 2/3
PAR-CLIP	photoactivatable- ribonucleoside-enhanced CLIP
PB	processing bodies
PCR	polymerase chain reaction
Pdcd4	programmed cell death 4
Perp	p53 apoptosis effector related to PMP22
PIG3	p53-induced gene 3
PIK3CA	phosphatidyl inositol-3-kinase, catalytic subunit alpha
PM-Scl75	polymyositis/scleroderma autoantigen 75 KDa
PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme
PRC2	polycomb repressive complex 2
PSF	polypyrimidine tract-binding protein (PTB)-associated splicing factor
PTB	polypyrimidine tract-binding protein
PTEN	phosphatase and tensin homolog

PTM	post-translational modification
PUMA	p53 up-regulated modulator of apoptosis
RBM5	RNA binding motif protein 5
RBP	RNA-binding protein
RE	response element
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RITA	reactivation of p53 and induction of tumor cell apoptosis
RMI1	recQ mediated genome instability 1
RNA	ribonucleic acids
RNA-FISH	ribonucleic acid - fluorescence in situ hybridization
RNPC1	RNA-binding region (RNP1, RRM) containing 1
ROBO2	roundabout, axon guidance receptor, homolog 1
RPL26	ribosomal protein L26
RRM	RNA recognition motif
SECIS	selenocysteine insertion sequence
Seq	sequencing
SF3A1	splicing factor 3a, subunit 1
SG	stress granules
siRNA	small interfering RNA
SOX2	SRY (sex determining region Y)-box 2
SPI1	spleen focus forming virus (SFFV) proviral integration oncogene
TARBP2	trans-activation-responsive RNA-binding protein 2
TERC	telomerase RNA component
TF	transcription factor
TIA1	T-cell-restricted intracellular antigen-1
TIAL1/TIAR	TIA1 cytotoxic granule-associated RNA-binding protein-like 1
TNF	tumor necrosis factor
TP53	tumor protein p53
TP63	tumor protein p63
TTP	tristetraprolin

uORF	upstream open reading frame
UTR	untranslated region
VEGF	vascular endothelial growth factor
Wig-1	wild-type p53 induced gene 1
WNT1	wingless-type MMTV integration site family, member 1
Xnr1	5'-3' Exoribonuclease 1
YB1	Y box binding protein 1
ZMAT3	zinc finger, matrin-type 3

1 INTRODUCTION

1.1 CANCER – A BRIEF OVERVIEW

Cancer is a widespread term used to indicate diseases in which abnormal cells divide in an uncontrolled way and are able to invade other tissues (metastasis). Many people consider cancer as one disease. In reality, there are about 200 known types of cancer. They differ in behavior, in growth rate, in their response to treatment and in their genetic signatures. But they also have common features which have been summarized by Hanahan and Weinberg in [1]. In summary cancer cells can: (1) stimulate their own growth; (2) ignore anti-proliferative signals; (3) resist their own programmed cell death (apoptosis); (4) stimulate the growth of blood vessels to supply nutrients to tumors (angiogenesis); (5) multiply endlessly; and (6) invade confined tissue and spread to distant sites (metastasis). Obtaining the last of these features is what distinguishes a tumor form being "benign" to being "malignant". Moreover, Hanahan and Weinberg published in 2011 an updated review including four additional cancer hallmarks [2]. The new list includes also: (7) the ability to deregulate normal cell metabolic processes; (8) the ability to evade the immune system surveillance; (9) genomic instability and high mutation rate; and (10) the ability to induce tumor-associated inflammatory response, which paradoxically promotes tumor progression rather than inhibition.

To answer the question "Why do people get cancer?" we should start by saying that the average human body is made up of about sixty trillion cells. Some of these cells divide regularly to replace damaged cells in an organized and controlled way. Indeed, our body forms billions of new cells every day after a division of a preexisting cell and from time to time mistakes happen – i.e. DNA mutations. These errors are not that uncommon, but thankfully, our immune system recognizes these cells and repairs or eliminates them. Moreover, a cell doesn't become cancerous overnight or as result of a single mutation. Instead, it is estimated that a normal cell needs to accumulate up to 10 "driver" mutations, meaning mutations that confer growth advantage on the cells carrying them, in order to become a cancer cell [3]. About 500 of the ~20,000 protein coding genes in the human genome have been found to be mutated and seem to contribute to cancer development [4].

Two of the main categories of genes that play a role in cancer are oncogenes and tumor suppressor genes. Oncogenes are those genes that typically control cell proliferation and if mutated can push cell cycle forward and also rescue cells from undergo apoptosis. Tumor suppressors on the other hand are those genes that restrict cellular growth and division and if mutated they prevent cells to stop from growing and to respond to pro-apoptotic signals [1]. According to The Cancer Genome Atlas (http://cancergenome.nih.gov), commonly mutated or altered oncogenes comprise KRAS (mutated in 95% of pancreatic adenocarcinomas and 40% to 50% of colorectal tumors), PIK3CA (mutated in 49% of uterine corpus endometrial

carcinoma and 34% of breast carcinomas), Myc (translocated in all cases of Burkitt's lymphomas and amplified in 42% of ovarian cancers) and BRAF (mutated in 60% of melanomas and thyroid carcinomas). Commonly mutated tumor-suppressors include TP53 (mutated in 94% of ovarian carcinomas and 90% of lung squamous cell carcinoma), CDKN2A (loss in about 66% of glioblastomas and 54% of bladder carcinomas), PTEN (loss in 40% of prostate cancer and mutated in 64% of Uterine Corpus Endometrial Carcinoma) and BRAC1 (mutated in about 50% of all hereditary breast cancer).

1.2 WIG-1 – GENE AND PROTEIN STRUCTURE

Wig-1 (for wild-type p53-induced gene 1, also known as *ZMAT3* or *PAG608*) is an RNA binding protein identified as a p53 transcriptional target gene in 1997. Through a PCR-based differential display technique Wig-1 mRNA was found to be induced by wild type p53 expressed from the temperature-sensitive mutant p53 construct in mouse [5] and in rat [6]. Four years later the human Wig-1 was also cloned and found to be located in chromosome 3q26.32 (chr3: 178735011-178789656 – hg19) [7, 8]. There are two transcript variants of the Wig-1 gene in human. Isoform 1 (NM_022470) is 8995 nucleotides long, contains 6 exons and corresponds to a protein of 289 amino acids. Isoform 2 (NM_152240) is 9113 nucleotides long, contains 7 exons and corresponds to a protein of 288 amino acids.

The two isoforms have different 5'UTR sequences: the first isoform has a 331 bps long 5'UTR while the second isoform has a 452 bps 5'UTR and contains an IRES element (361-452) suggesting that the translation of the two isoforms might be differently regulated. Moreover, Wig-1 contains two in-frame ATG and one upstream out-of-frame ATG. The full-length protein is translated from the first in-frame ATG; twenty amino acids downstream of it, the second in-frame ATG can also be used for translational initiation. This can happen if the upstream out-of-frame ATG is used, giving rise to an uORF that terminates in between the first and second in-frame ATG, thus allowing the translation of the shorter of the Wig-1 species that lacks the first 20 amino acids. These two Wig-1 species are both detectable and clearly visible on Western blot analysis using an antibody raised against the full length protein; moreover, it seems that they might have distinct functions, as supported by the fact that their expression changes depending on the conditions, for example after p53-dependent Wig-1 induction upon stress by DNA damaging agents (Hellborg et al, unpublished results).

Human Wig-1 has a perfect consensus p53 response element (RE) on intron 1 (2455 bps upstream of the translation start codon). See Figure 1. Induction of p53 by doxorubicin and Nutlin in U2OS cells or RITA, Nutlin and 5-Fu in MCF7 cells lead to p53 binding to its RE on Wig-1 gene (Chip-Seq analysis) and increase in Wig-1 expression levels [9, 10].

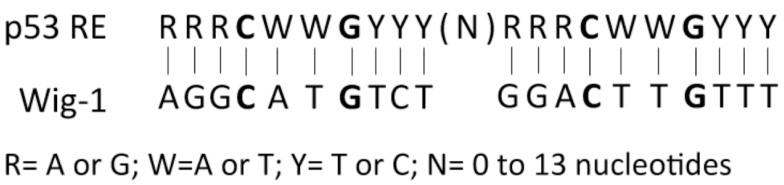

Figure 1: Sequence of the p53 response element on Wig-1 promoter

The Wig-1 3'UTR is 7794 bp long and contains five polyadenylation (poly(A)) signals (position 1581, 4950, 6771, 7283, 7764), fourteen AU-rich elements (ARE), a SECIS type I and a SECIS type II, a BRD-Box, a GY-Box, an AluSx and AluY (Figure 2). All these elements are important RNA regulatory motifs and they will be explained in detail in paragraph 1.5.

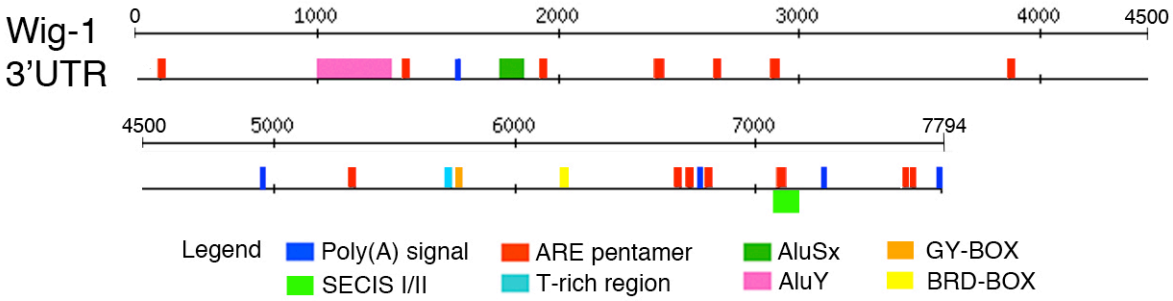

Figure 2: Regulatory elements embedded on Wig-1 3'UTR

The Wig-1 protein contains a nuclear localization signal (aa 194-210) and three zinc fingers (ZFs) of the Cys2His2 type (aa 72-94, 149-171, 247-269). The ZFs are characterized by an uncommon inter-histidine distance (five amino acids instead of the usual three to four). Additionally, the distance between the ZFs is also longer than what observed for other ZFs (56-75 amino acids instead of the common six to eight) (Figure 2a).

Wig-1 is a highly conserved protein, especially the zinc-fingers that are almost completely conserved from human to amoeba, a unicellular eukaryote that diverged from human lineage about 1.5 billion years ago [11, 12] (Figure 2b). Human and mouse Wig-1 share 87% identity in the whole protein sequence, 100% identity in the first and second zinc fingers and 97% identity in the third zinc-finger. The zinc-fingers are the regions that show higher conservation among various species (http://www.uniprot.org) [12, 13].

Wig-1 zinc fingers are characterized by an unusual structure that is comparable to those of a small group of double stranded RNA (dsRNA) binding protein named JAZ. JAZ was the founder of a new class of C2H2-type zinc finger proteins; it has almost no affinity for DNA or single-stranded RNA, but binds preferentially to double-stranded RNA (dsRNA) [14]. The

structural similarity between Wig-1 and JAZ led to the discovery that Wig-1 is also an RNA binding protein [15].

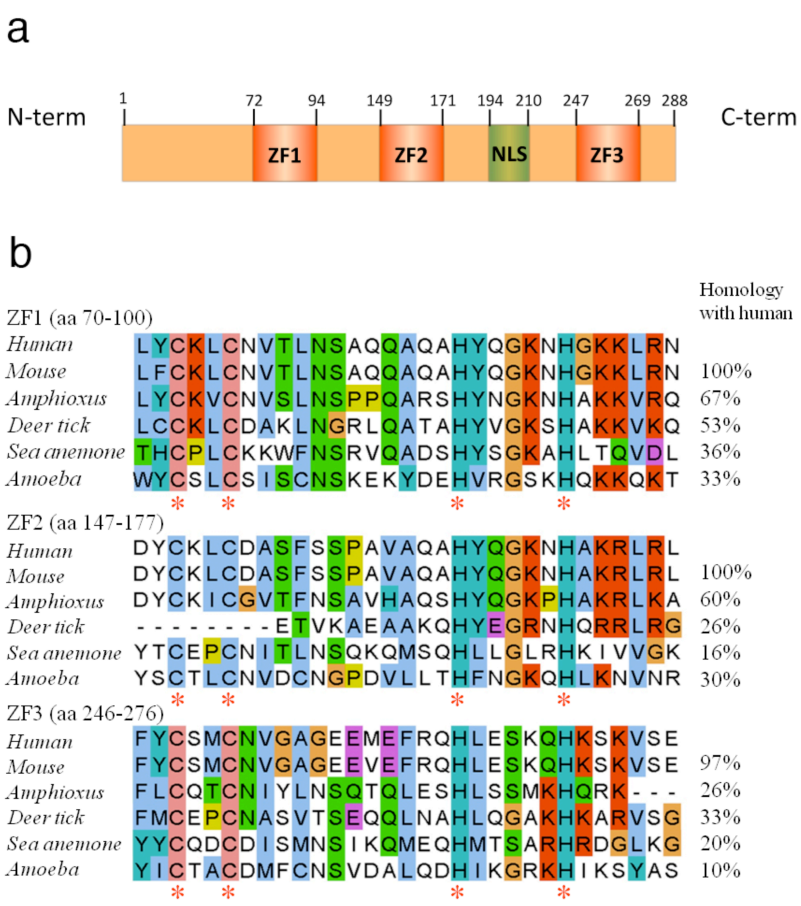

Figure 3: Wig-1 protein structure and Zinc-Fingers alignments

1.3 WIG-1 PROTEIN FUNCTION

Wig-1 is a RNA binding protein that preferentially binds double-stranded RNA (dsRNA). Mutational studies showed that the first and second zinc fingers of Wig-1 are required to maintain dsRNA binding [5, 13]. Wig-1 binds to dsRNA longer than ≥ 23 bps, although it can also bind shorter siRNA/miRNA-like (carrying 2 nt 3′-protruding ends) dsRNA (21bps) in vitro, but with less affinity [16].

As previously mentioned Wig-1 is a transcriptional target of the p53 tumor suppressor and this suggests that it might be involved in the p53 response to cellular stress. Indeed, Wig-1 inhibits cell growth in a colony formation assay [8, 16]. Remarkably, both Wig-1 overexpression and knockdown has a negative effect on cell growth indicating that Wig-1 levels are finely regulated and maintained well-balanced [17].

Wig-1 protein has been shown to interact with a number of other proteins [17-21]. Table 1 summarizes these data.

Table 1: List of Wig-1 interacting proteins

Interacting protein	Description	Method	Function	Reference
ACTA2	actin, alpha 2, smooth muscle, aorta	MS	Glomerular mesangial cell development, Regulation of blood pressure, Response to virus, Vascular smooth muscle contraction	Huttlin, 2014
DICER1	dicer 1, ribonuclease type III	MS	RNA-mediated gene silencing	Huttlin, 2014
SF3A1	splicing factor 3a, subunit 1, 120kDa	MS	mRNA proscessing and splicing	Huttlin, 2014
SUPV3L1	ATP-dependent RNA helicase SUPV3L1, mitochondrial	MS	mitochondrial RNA metabolism	Huttlin, 2014
DCAF17	DDB1 and CUL4 associated factor 17	MS	Protein ubiquitination	Huttlin, 2014
ISCA1	iron-sulfur cluster assembly 1	MS	maturation of mitochondrial 4Fe-4S proteins	Huttlin, 2014
TARBP2	TAR (HIV-1) RNA binding protein 2	MS	RNA-mediated gene silencing, Translation regulation	Huttlin, 2014
FTO	Alpha-ketoglutarate-dependent dioxygenase FTO	MS	DNA damage, DNA and RNA repair	Huttlin, 2014
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	MS	Transcription regulation, Aortic smooth muscle cell differentiation, Chromatin remodeling, Nervous system development	Tando T, 2010
APP	amyloid beta (A4) precursor protein	Protein Array	Cell surface receptor, regulation of neuronal adhesion, axonogenesis and cell mobility, Transcription regulation	Olah J, 2011
SUMO2	small ubiquitin-like modifier 2	MS	Regulation of proteasomal ubiquitin-dependent protein catabolic process	Ouyang J, 2009
DHX9	ATP-dependent RNA helicase A	Co-IP	Transcriptional activator	Prahl M, 2008
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	Co-IP	mRNA processing and splicing, RNA transport	Prahl M, 2008

1.4 TRANSCRIPTIONAL AND EPIGENETIC WIG-1 REGULATION

Human Wig-1 has a perfect consensus p53 RE on its first intron (see above). Moreover, it has been shown that BRD7 transcriptional co-factor is necessary for p53 transcriptional activation of Wig-1 and that BRD7 knockdown lead to decreased Wig-1 levels [22]. Additionally, data extracted from the Encyclopedia of DNA Elements (ENCODE) Consortium, an international collaboration of research groups funded by the National Human Genome Research Institute (NHGRI), revealed that Wig-1 promoter is bound by at least 16 more transcription factor (Table 2) [23]. These data, together with the evidence that Wig-1 is expressed at high levels also in cells lacking p53, indicate that Wig-1 is not merely a target of the p53 tumor suppressor. Possibly, one or more of these transcription factors might regulated Wig-1 expression in different conditions and maybe be tissue specific.

Wig-1 promoter has been shown to be target for CpG methylation, and this modification is suggested to be the cause of Wig-1 downregulation in KM-H2 Hodgkin Lymphoma cells and in gastric cancer cell line infected by Epstein-Barr virus [24, 25].

Recent studies reveal that Wig-1 is also regulated epigenetically. ChIP-Seq analysis performed in mESC in two independent studies shows that Wig-1 is a target of the histone

methyl transferase PRC2 on H3K27me3 [26, 27]; indeed PCR2 silencing results in Wig-1 upregulation [27]. A key function of PRC2 in mammals is to regulate stem cell function, where it can promote self-renewal through direct repression of pro-differentiation genes [28].

Table 2: List of transcription factor binding to Wig-1 promoter (ChIP-Seq data)

TF	# of experiments	Cell Type	Source
CTCF	97	A549, AG04449, AG04450, AG09309, AG09319, AG10803, AoAF, BJ, Caco-2, Dnd41, ECC-1, Fibrobl, Gliobla, GM06990, GM12864, GM12865, GM12872, GM12873, GM12874, GM12875, GM12878, GM12891, GM12892, GM19238, GM19239, GM19240, H1-hESC, HA-sp, HAc, HBMEC, HCFaa, HCM, HCPEpiC, HCT-116, HEEpiC, HeLa-S3, HepG2, HFF, HFF-Myc, HMEC, HMF, HPAF, HPF, HRE, HRPEpiC, HSMM, HSMMtube, HUVEC, HVMF, IMR90, K562, MCF-7, NB4, NH-A, NHDF-Ad, NHDF-neo, NHEK, NHLF, Osteobl, ProgFib, RPTEC, SAEC, SK-N-SH_RA, SKNBE2_C, T-47D, WERI-Rb-1, WI-38	(a,b,c,d,e)
MAX	7	A549, GM12878, HeLa-S3, HepG2, K562, NB4	(d,e)
USF1	9	A549, GM12878, H1-hESC, HepG2, K562, SK-N-SH_RA	(e)
NFKB p65	6	GM12891, GM19099, GM18505, GM10847, GM12878, GM15510	(d)
EBF1	2	GM12878	(d,e)
EGR1	3	K562, GM12878	(e)
ELF1	3	K562, GM12878, HepG2	(e)
MAZ	1	GM12878	(d)
NRF1	5	GM12878, H1-hESC, K562, HeLa-S3, HepG	(d)
SPI1	3	GM12891, GM12878, K562	(e)
TCF12	2	GM12878	(e)
USF2	3	GM12878, HeLa-S3, HepG2	(d)
E2F1	3	MCF-7, HeLa-S3	(f)
E2F6	3	K562, HeLa-S3	(e,f)
CTCFL	1	K562	(e)
E2F4	1	K562	(f)
ETS1	1	K562	(e)

(a): Iyer's Lab - University of Texas at Austin; (b): Stamatoyannopoulous's Lab - University of Washington; (c): Bernstein's Lab - Broad Institute; (d): Snyder's Lab - Stanford University; (e): Myers Lab - Hudson Alpha Institute for Biotechnology; (f): Farnham's Lab - University of Southern California.

Cell lines: A549: Human lung adenocarcinoma epithelial cell line; AG04449: fetal buttock/thigh fibroblast; AG04450: fetal lung fibroblast; AG09309: adult toe fibroblast; AG09319: gum tissue fibroblasts ; AG10803: abdominal skin fibroblasts; AoAF: Aortic Adventitial Fibroblasts; BJ: foreskin fibroblast; Caco-2: colorectal adenocarcinoma; Dnd41: T cell leukemia with Notch mutation; ECC-1: endometrium adenocarcinoma; Fibrobl: child fibroblast; Gliobla: glioblastoma multiforme; GM(#): Epstein-Barr Virus transformed B-lymphocyte; H1-hESC: embryonic stem cells; HA-sp: astrocytes spinal cord; HAc: astrocytes-cerebellar; HBMEC: brain microvascular endothelial cells; HCFaa: cardiac fibroblasts- adult atrial; HCM: cardiac myocytes; HCPEpiC: choroid plexus epithelial cells; HCT-116: colorectal carcinoma ; HEEpiC: esophageal epithelial cells; HeLa-S3: cervical carcinoma; HepG2: hepatocellular carcinoma; HFF: foreskin fibroblast; HFF-Myc: foreskin fibroblast cells expressing canine cMyc; HMEC: mammary epithelial cells; HMF: mammary fibroblasts; HPAF: pulmonary artery fibroblasts; HPF: pulmonary fibroblasts isolated from lung tissue; HRE: renal epithelial cells; HRPEpiC: retinal pigment epithelial cells; HSMM: skeletal muscle myoblasts; HSMMtube: skeletal muscle myotubes differentiated from the HSMM cell line; HUVEC: umbilical vein endothelial cell; HVMF: villous mesenchymal fibroblast cells; IMR90: fetal lung fibroblasts; K562: myelogenous leukemia; MCF-7: mammary gland, adenocarcinoma; NB4: acute promyelocytic leukemia cell line; NH-A: astrocytes; NHDF-Ad: adult dermal fibroblasts; NHDF-neo: neonatal dermal fibroblasts; NHEK: epidermal keratinocytes; NHLF: lung fibroblasts; Osteobl: osteoblasts; ProgFib: fibroblasts, Hutchinson-Gilford progeria syndrome; RPTEC: renal proximal tubule epithelial cells; SAEC: small airway epithelial cells; SK-N-SH_RA: neuroblastoma cell line differentiated with retinoic acid; SKNBE2_C: neuroblastoma; T-47D: mammary ductal carcinoma; WERI-Rb-1: retinoblastoma; WI-38: embryonic lung fibroblast cells, hTERT immortalized

Interestingly, Wig-1 promoter in mouse embryonic stem cells (mESC) harbors both the activating H3K4me3 mark and the repressive H3K27me3 mark. These bivalent domains are considered to be a feature of developmentally regulated genes. They are maintained repressed until the time when a particular differentiation signal will switch the activating mark on and engage the cell to develop into a more specific functional type. Following differentiation, bivalent promoters embrace either an active or a repressed state, depending on cell fate. [26, 29]. Remarkably, Wig-1 promoter in neural progenitor cells and embryonic fibroblasts carries H3K4me3 only [29]. Another study in mESC shows that Wig-1 has the classical methylation profile of genes expressed at high levels (positive H3K4me3, H3K36me3, and negative H3K27me3) [30].

PCR2 is also linked to pathogenesis of human cancer and it is commonly overexpressed or mutated in cancer [31]. Consistent with this notion, Wig-1 gene shows higher H3K27me3 methylation in MLL-leukemia cell line as compared to 32D myeloblasts cells [27].

Le Martelot et al. performed genome-wide analysis of the locations of RNA polymerase II (Pol II) and the epigenetic histone modifications H3K4me3 and H3K36me3 at specific times of the day, relating these data to mRNA expression levels. They show that Pol II transcriptional rhythms are biphasic in mouse liver, having predominant peak activities in the morning and evening. The analysis places Wig-1 among the class of core circadian clock genes, defined as genes whose protein products are necessary components for the generation and regulation of circadian rhythms. Wig-1 showed rhythmicity both in transcriptional and mRNA accumulation and its mRNA showed to be short-lived [32].

Altogether these evidences suggest a tight epigenetic regulation of Wig-1 expression. This regulation appears to be important for the modulation of Wig-1 function during development, differentiation and possibly carcinogenesis, but also, in the physiological regulation of circadian rhythms.

1.5 POST-TRANSCRIPTIONAL WIG-1 REGULATION

Not much is known about regulation of Wig-1 at mRNA level, although the presence of numerous regulatory motifs on it 3'UTR suggests that it might be target of a number of RNA-binding proteins. As mentioned above (see also Figure 2) Wig-1 3'UTR contains both a type I and type II SECIS. SECIS elements are cis-acting stem-loop RNA structures that are found in the 3′-untranslated regions of all eukaryotic and archaeal selenoprotein mRNAs [33]. Interestingly, Wig-1 mRNA is bound by the eukaryotic initiation factor 4a3 (eIF4a3) as shown by HITS-CLIP analysis (Table 4) [34]. eIF4a3 acts as a transcript-specific repressor of selenoprotein mRNA translation during selenium deficiency [35]. Although no previous reports indicate Wig-1 as a potential non-essential selenoprotein, these data advise differently and it certainly deserves further investigation. Additionally, the Wig-1 3'UTR also contains a

GY-box (GUCUUCC) and the Brd-box (AGCUUUA) (Figure 2). These 7-nt motifs, found originally in *Drosophila melanogaster,* are known to mediate negative post-transcriptional regulation [36, 37]; they are broadly distributed in the 3′ UTRs of Notch genes and function to constrain their activity during normal development of *D. melanogaster* nervous system. A study demonstrated that GY-box-containing 3′ UTRs are inhibited by miR-7 and those with Brd-boxes by miR-4 and miR-79 [38]. miRNA-mediated regulation may be a conserved feature of Notch target genes in human according to [38], thus it is plausible and would be intriguing to investigate whether Wig-1 is also included in this scenario. RNA editing by adenosine deamination is a relatively common post-transcriptional alteration of mRNA catalyzed by the ADAR family of enzymes, which recognize the repetitive retrotransposable Alu element on the UTR of genes. Alu elements are also involved in the regulation of splicing [39, 40] and translation [41-43] of those transcripts that bear them, both positively and negatively. Wig-1 possesses two of these elements, namely an AluSx, a Alu subfamily that show the highest levels of editing [44] and an AluY, the evolutionarily youngest Alu subfamily in primates. Up to now, there are no reports showing usage of this motifs in Wig-1 mRNA as target for RNA regulation or editing.

Table 3: List of miRNAs that interact with mouse or human Wig-1 3'UTR

	Wig-1 associated microRNA	Cell Type	Reference
Mouse	miR-539, miR-27, miR-191, miR-146	CD4+ T cells Brain tissue mESC	Loeb GB, et al. 2012 Helwak A, et al. 2013 Leung AK, et al. 2011
Human	miR-320, miR-4295, miR-129, miR-130, miR-613, miR-16, miR-15, miR-19, miR-539, miR-193, miR-195, miR-497, miR-454, miR-301, miR-1, miR-27, miR-4429, miR-124, miR-191, miR-206, miR-3666, miR-361, miR-503, miR-424	HeLa HEK293S BC-1 BC-3 hESCs HEK293 LCL-BACD3 EF3D-AGO2 LCL35	Xue Y, et al. 2013 Karginov FV, et al. 2013 Gottwein E, et al. 2011 Lipchina I, et al. 2011 Kishore S, et al. 2011 Memczak S, et al. 2013 Skalsky RL, et al. 2012 Hafner M, et al. 2010

HeLa: cervical carcinoma; HEK293S: Adenovirus 5 transformed human embryonic kidney cells; BC-1: Lymphoma cells; BC-3: Lymphoma cells; hESCs: human embryonic stem cells; HEK293: human embryonic kidney cells; LCL-BACD3: EBV B95-8-infected lymphoblastoid cells; EF3D-AGO2: EBV B95-8-infected lymphoblastoid cells stably expressing Flag-AGO2; LCL35: EBV B95-8-infected lymphoblastoid cells.

In a study where Wig-1 mRNA was induced by p53 in HT29-tsp53 cells at 32°C (active p53), they showed that Wig-1 mRNA expression levels decreases at least eightfold within 6 h after the temperature shift to 38°C (inactive p53), indicating that Wig-1 mRNA half-life is lower that 2 h. This instability was found to correlate with the high content in AU-rich element of Wig-1 3'UTR (14 AUUUA pentamers and a 12 nts long T-stretch – Figure 2) [45]. Wig-1 stability curve and 3'UTR composition was similar to those of the pro-arrest p21 gene, for which, regulation of stability on the 3'UTR by a number of RBPs has been reported [46, 47].

These data also indicate that the transcriptional induction of short-lived mRNAs like Wig-1 is important to modulate the p53 response. Interestingly, other AU-rich binding proteins have been reported to bind to Wig-1 mRNA (Table 4), namely the stabilizing proteins hnRNP-C [48] and HuR [49] and the destabilizing protein FMRP [50] and TIAR [51].

Table 4: List of RBPs associated to Wig-1 mRNA [34, 52, 54, 59, 64-74]

RNA-Binding Protein name	# of target sites on Wig-1 mRNA	# of experiments	Reference	Cell type	Method
CAPRIN1	3	1	Baltz AG, 2012	HEK293	PAR-CLIP
DGCR8	2	1	Macias S, 2012	HEK293T	HITS-CLIP
eIF4AIII	37	2	Saulière J, 2012	HeLa	HITS-CLIP
EWSR1	4	1	Hoell JI, 2011	HEK293	PAR-CLIP
FMRP	84	4	Ascano M Jr, 2012	HEK293	PAR-CLIP
FUS	21	4	Nakaya T, 2013; Hoell JI, 2011	Brain tissue; HEK293	HITS-CLIP; PAR-CLIP
FUS-mutant	6	1	Hoell JI, 2011	HEK293	PAR-CLIP
FXR1	3	1	Ascano M Jr, 2012	HEK293	PAR-CLIP
FXR2	7	1	Ascano M Jr, 2012	HEK293	PAR-CLIP
hnRNPC	35	1	Zarnack K, 2013	HeLa	iCLIP
HuR	15	1	Kishore S, 2011	HEK293	PAR-CLIP
IGF2BP1	15	1	Hafner M, 2010	HEK293	PAR-CLIP
IGF2BP2	21	1	Hafner M, 2010	HEK293	PAR-CLIP
IGF2BP3	18	1	Hafner M, 2010	HEK293	PAR-CLIP
LIN28A	1	1	Hafner M, 2010	HEK293	PAR-CLIP
LIN28B	6	1	Hafner M, 2010	HEK293	PAR-CLIP
MOV10	4	1	Sievers C, 2012	HEK293	PAR-CLIP
PTB	20	1	Xue Y, 2013	HeLa	HITS-CLIP
PUM2	2	1	Hafner M, 2010	HEK293	PAR-CLIP
SFRS1	1	1	Sanford JR, 2009	HEK293T	HITS-CLIP
TDP43	1	1	Tollervey, 2011	brain tissue; SH-SY5Y; H9 hESC	iCLIP
TIAL1	1	1	Wang Z, 2010	HeLa	iCLIP
UPF1	60	3	Zünd D, 2013	HeLa	iCLIP
ZC3H7B	6	1	Baltz AG, 2012	HEK293	PAR-CLIP

HEK293:Human Embryonic Kidney 293 cells; HEK293T: SV40 large T antigen transformed HEK293; HeLa: cervical carcinoma; SH-SY5Y: neuroblastoma cell line; H9 hESC: human embryonic stem cells; PAR-CLIP: Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation; HITS-CLIP: High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation; iCLIP:individual-nucleotide resolution Cross-Linking and ImmunoPrecipitation.

Next generation sequencing methods, particularly AGO proteins PAR- and HITS-CLIP, allowed to detect 24 miRNAs in human (in at least 5 out of 8 independent studies) [52-59] and 4 miRNAs in mouse (in at least 2 out of 3 independent studies) [60-62] as potential Wig-1 mRNA targeting miRNAs (Table 3). Another study shows that miR-206 might be involved in the regulation of Wig-1 expression in glioblastoma cells (U373, GBM2, GBM5) [63].

Splicing might also affect Wig-1 expression and modulate it function in different context. It has been shown that Wig-1 alternative splicing is higher in neoplastic colonic epithelial cells than in healthy epithelial cells [75].

1.6 POST-TRANSLATIONAL WIG-1 REGULATION

Wig-1 protein carries a number of post-translational modifications (PTMs) as shown by mass spectrometry data collected in our lab (Xu L. et al, Unpublished data). Figure 4 summarizes these findings. Interestingly, some sites differ in their phosphorylation status in unstressed as compared to stressed condition (cisplatin treatment), supporting the belief that Wig-1 plays a role in the cell response to stress and that PTMs might affect Wig-1 behavior in different conditions. Preliminary results from Co-IP experiments performed with phosphor- or acetylated antibody has confirmed part of the results found by MS analysis (Xu L. et al, Unpublished data).

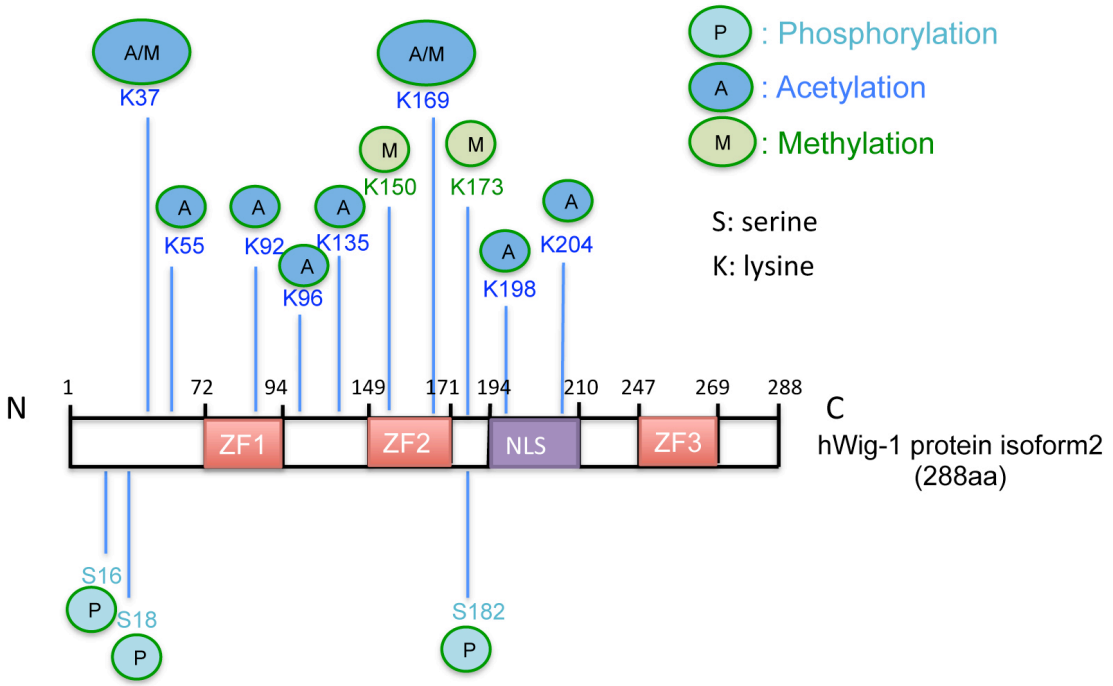

Figure 4: Wig-1 protein post-translational modifications identified by MS. Picture and data courtesy of Lidi Xu.

1.7 WIG-1 IN PHYSIOLOGY AND DISEASE

1.7.1 Wig-1 in cancer

Human WIG-1 maps to 3q26.32 [8], a region that is commonly amplified in cancer and contains several genes with relevance to cancer, such as the phosphoinositide-3-kinase catalytic alpha polypeptide gene (PIK3CA) [76], the telomerase RNA component gene (TERC) [77, 78], the TP63 gene [79], and the sex-determining region Y-box 2 gene (SOX2) [80-82]. A recent study shows that Wig-1 is upregulated in 10 out of 10 tested cancers carrying wtp53 (breast carcinoma, colorectal cancer, glioblastoma, endometrial carcinoma, bladder carcinoma, ovarian serous adenocarcinoma, acute myelogenous leukaemia, lung adenocarcinoma, stomach adenocarcinoma and low-grade glioma) [83]. Contrarily, mutant p53 seems to exert a dominant negative effect over Wig-1 expression; indeed Wig-1 is expressed at lower levels in cells expressing mutant p53 (R273H, R249S, R175H and R280K) [84, 85]. In another investigation Wig-1 expression was tested in 82 melanoma metastases and found to be 6.7 fold lower compared to 8 melanocyte cell lines; in this case, the difference was not related to the p53 status of these tumors [86].

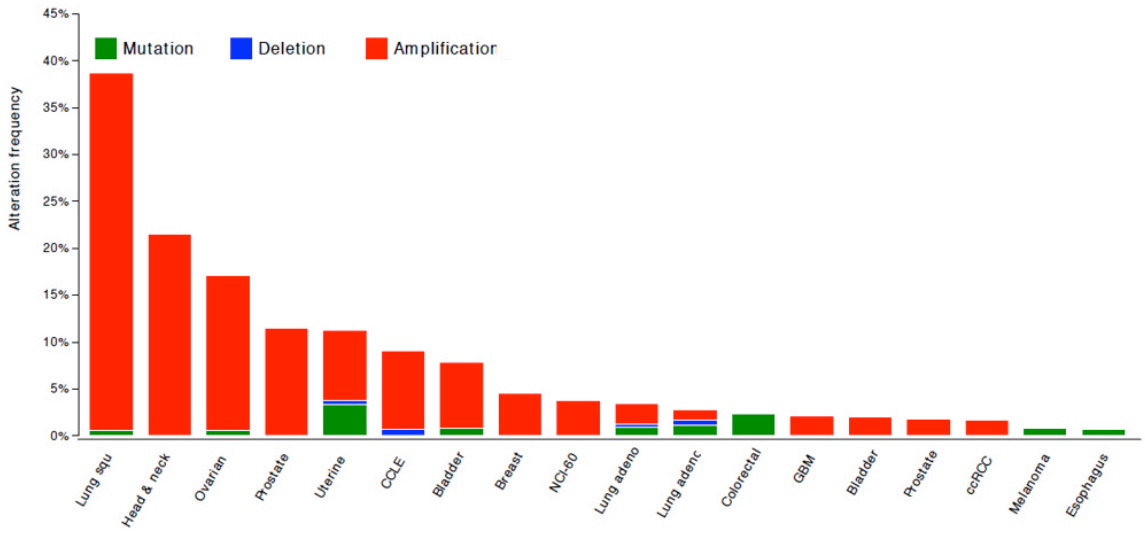

Figure 5: Wig-1 alteration status in human cancers. Data issued from TCGA portal analyses. Only published results are shown in this graph.

Through the visualization of the cancer genomic data stored in the cBioPortal for Cancer Genomics [87, 88], it is evident the level of Wig-1 alteration in different cancers. Figure 5 shows Wig-1 status in: lung squamous cell carcinoma [89], head and neck squamous cell carcinoma, ovarian serous adenocarcinoma [90], prostate adenocarcinoma [91, 92], uterine

corpus endometrioid carcinoma [93], cancer cell line encyclopedia [94], breast invasive carcinoma [95], NCI-60 cell lines [96], lung adenocarcinoma [97], colorectal adenocarcinoma [98], glioblastoma [99], bladder urothelial carcinoma [100, 101], kidney renal clear cell carcinoma [102], melanoma [103], esophageal adenocarcinoma [104]. Figure 5 reveals that Wig-1 is mostly amplified and very rarely deleted.

Despite these data, a study focused on the analysis of Wig-1 expression in cervical carcinoma cell lines and patient tumor biopsies (squamous cervical carcinomas and adenocarcinomas) revealed that Wig-1 is not the critical gene that drives 3q gain in cervical cancer [105]. This indicates that additional studies are necessary in order to evaluate how relevant is Wig-1 amplification for tumor formation and/or progression. Nevertheless, considering that Wig-1 is required in order to maintain cell proliferation, it is plausible to think that the tumor might benefit from its increased expression somehow.

Figure 6 shows the location and type of mutations found on Wig-1 gene in the cancers tested in Figure 5. Similarly to what previously discussed about the relevance of Wig-1 amplification, further investigation is needed in order to determine whether these alterations are passenger (i.e. a random and natural consequence of cancer's elevated mutation rate) or driver (i.e. cancer-causing) mutations.

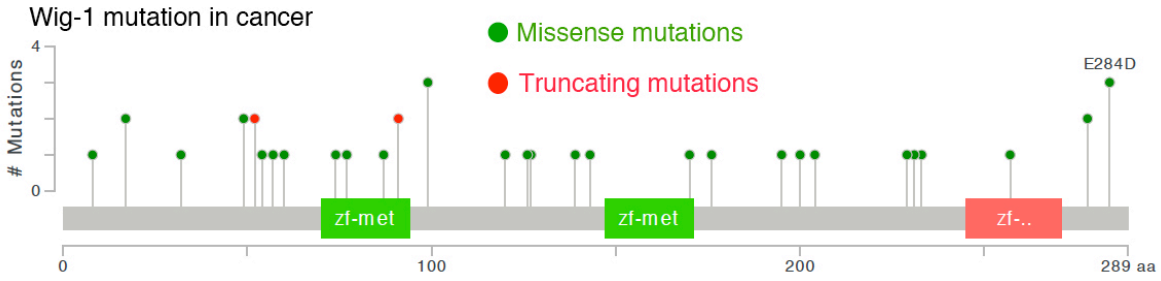

Figure 6: Wig-1 gene mutations found in human cancers. Data issued from TCGA portal analyses.

1.7.2 Wig-1 in stem cell maintenance and differentiation

Wig-1 is expressed at higher levels in haematopoietic, neuronal and embryonic stem cells compared to the corresponding differentiated cell types [106]. Depletion of Bmi-1, a factor required for maintenance of adult self-renewing haematopoietic stem cells, led to upregulation of Wig-1 in these cells [107]. These data suggest a role of Wig-1 in stem cell maintenance. Besides, the fact that Wig-1 mRNA is upregulated (84 fold change) by the nerve growth factor (NGF), a neurotrophin essential for neuronal differentiation, indicates that it might also play a role in cell differentiation [108]. Additionally, Wig-1 promoter in mouse embryonic stem cells harbors both the activating H3K4me3 mark and the repressive H3K27me3 mark. These bivalent domains are believed to control the expression of

developmental genes, maintaining repression in the absence of differentiation signals [26, 29]. Table 2 shows a list of TFs that bind to Wig-1 promoter. Among them, SPI1, a factor with a distinctive and indispensable role in maintenance of hematopoietic stem cells and their differentiation [109]. Moreover, ETS1 and ELF1 has been identified as potent TF for ESC differentiation toward blood cells [110]. MAX has been recently proposed as repressor of germ cell-related genes in embryonic stem cells [111]. Last but not least, p53, confirmed Wig-1 activator, has been extensively reviewed for its role in embryonic, adult and induced pluripotent stem cell regulation [112-115]. Unpublished results from Vilborg et al., shows that homozygous Wig-1 mice knockout is associated with lethality before the blastocyst stage thus emphasizing the possibility that it has indeed a determining role in stem cell maintenance and differentiation [12].

1.7.3 Wig-1 in the brain

A number of studies indicate that Wig-1 has a role in the physiology of the brain, as its deregulation is associated with different neurological disorders. Wig-1 is abundantly expressed in the central nervous system [5] and its expression increases in the hippocampus following transient cerebral ischemia [116-119]. The rat Wig-1 homologous, PAG608, is induced in motor neurons of L-DOPA-injected hemi-parkinsonian rats [120]. Its expression is also increased in lumbar spinal cord motor neurons of transgenic mouse model of amyotrophic lateral sclerosis (ALS) [121] and it has been proposed as a marker for ALS onset. Moreover, Wig-1 is among the genes that are downregulated in VEGF-/- mice at the age of five month, the time when these mice develop a motor neurodegenerative phenotype that resemble ALS [122]. Additionally, PC12 cells (neural crest origin) treated with NGF, a neurotrophin essential for neuronal differentiation, lead to increase in Wig-1 gene expression by eighty-four fold change [108]. Sedaghat et al, performed a microarray analysis in mouse brain after Wig-1 knockdown using highly specific antisense oligonucleotides (ASOs) and found a 50% significant reduction in mutant huntingtin protein, but also proteins such as the autism susceptibility candidate 2 (AUTS2), linked to neuropathy of autism, the roundabout axon guidance receptor homolog 2 (ROBO2), critically important for axon guidance and in CNS development, the inner mitochondrial membrane peptidase 2-like (IMMP2L), a mitochondrial peptidase that has been linked with Autism Spectrum Disorders (ASDs) and with Tourette Syndrome [123]. ChIP-Seq analysis revealed that Wig-1 is one of the target of the nuclear respiratory factor 1 (NRF1), a TF whose deregulation contributes to neurodegenerative processes underlying Parkinson's, Alzheimer's and Huntington's disease [124]. Also, a protein arrays used for the identification of Tau/β-amyloid protein interactors on large scale (main constituent of amyloid plaque in the brains of Alzheimer's patients) indicated Wig-1 has one of the partners [18].

1.8 THE p53 TUMOR SUPPRESSOR

The p53 protein (also known as tumor protein 53, *TP53*) is a nuclear transcription factor [125, 126] and one of the most important tumor suppressors, as proved by the fact that the inactivation of the p53 network is required for the development of nearly all human cancers (http://p53.fr) [127]. The transcriptional activity of p53 is crucial for its tumor prevention and suppression function [128]. p53 binds DNA in a sequence specific manner (Figure 1 shows p53 response element (RE)) [129]. A transcription-independent induction of apoptosis by p53 has been reported [130-132], however the tumor suppression function of p53 is primarily due to its role as a transcription factor (Figure 7).

Up to now, as many as 45,000 p53 somatic gene mutations have been reported [127], designating p53 as the most frequently mutated gene in human tumors. Moreover, a germline mutations of p53 in Li-Fraumeni syndrome patients, characterized by very high cancer susceptibility, provided a straight link between tumor development and p53 mutation [133]. These observations demonstrate the critical role of p53 in tumor prevention. In addition to p53 gene mutations, other mechanisms such as viral infection or alterations of key regulators of p53 activity can also lead to its inactivation. For example, certain DNA viruses (e.g. SV40, HPV and adenoviruses) encode proteins that bind and target p53 for degradation [134]. Additionally, MDM2 and MDM4, two negative regulators of p53, are amplified in a variety of cancers [135], while the p53 upstream positive regulator ARF/p14^{ARF} gene can be deleted or epigenetically inactivated [136].

In unstressed cells, p53 protein is maintained at very low levels by Mdm2, an E3 ubiquitin ligase that promotes p53 degradation; the Mdm2 gene is positively regulated by p53, defining a negative feedback loop that controls p53 activity. Cellular stress releases the inhibitory effects of Mdm2, thus triggering p53 stabilization and activation. Once activated, p53 exerts its protective function by repairing damaged DNA and inhibiting the proliferation of potentially tumorigenic cells through induction of cell cycle arrest, senescence or apoptosis (Figure 7) [137]. The p53 response is elicited by a variety of stress signals such as DNA damage, oncogene activation, abnormal mitosis, loss of cell–cell contact, nutrient deprivation, telomerase shortening and hypoxia [137]. p53 is a powerful transcriptional regulator able to regulate the expression of hundreds of genes [137, 138]. To add more complexity, p53 interacts with several cofactors and binding partners affecting its transcriptional activity. Moreover, the other p53 family members, p63 and p73, can also modulate transcription and share a number of target genes with p53 [139-142].

Three major post-translational modifications (ubiquitination, phosphorylation and acetylation) are responsible for regulating the activity and stability of p53 [143-145] and altogether affect the precise and selective behavior of p53.

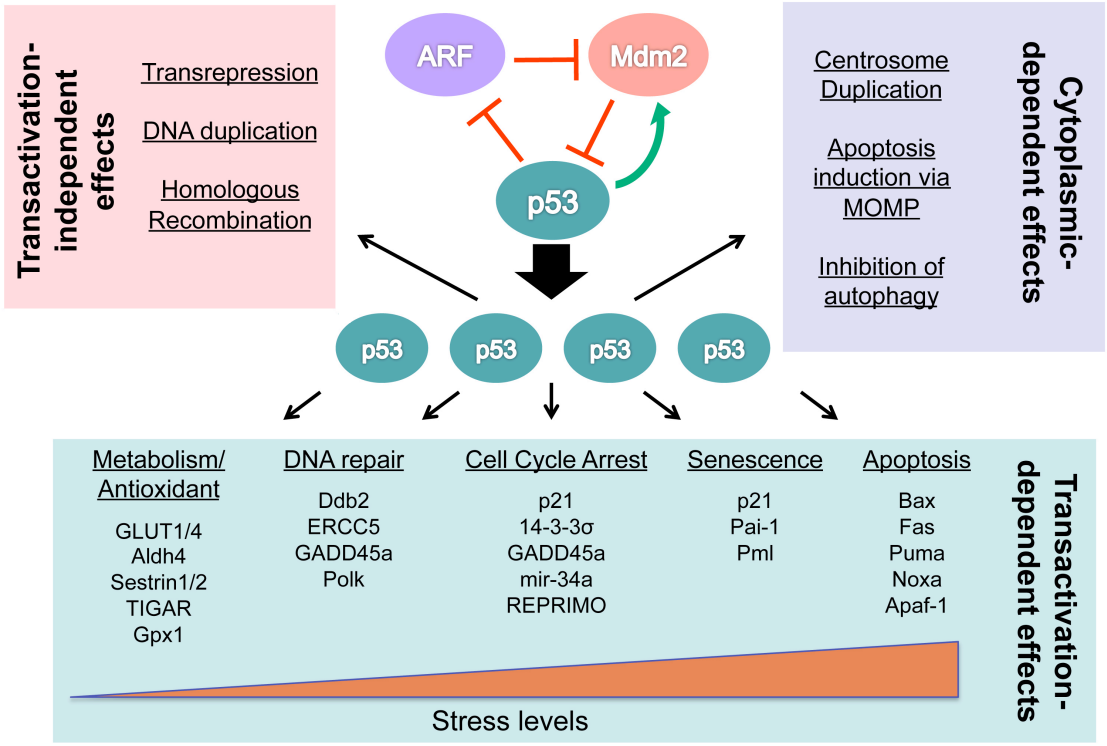

Figure 7: Schematic representation of p53 functions

1.8.1 p53 and cell fate decision

Understanding the molecular basis of p53 selectivity among its distinct transcriptional targets is crucial in the comprehension of how p53 chooses between life or death for the cell. The ability of p53 to display a varied range of responses to stress and what factors are modulating the choice have been investigated for some time. p53 is a cellular stress sensor; in response to mild reparable damage, p53 triggers transient cell cycle arrest allowing the necessary time for repair of the damage and cell cycle re-entry. Differently, severe or irreparable DNA damage will often lead to apoptosis (Figure 7) [146]. It is known that promoter-selective transactivation of p53 targets is crucial for the diverse cellular responses to distinct stresses. p53 transcription selection depends on several mechanisms such as post-translational modification of p53, interaction with different binding partners and gene specific chromatin modification. For example, DNA damage induces p53 phosphorylaytion at serine 46 by several kinases such as HIPK2, DYRK2 and p38 [147-149]. This modification turns p53 into a conformation that is more efficient at transactivating the expression of the proapoptotic target genes p53AIP1, Noxa, Perp, and PUMA [150, 151], while there is no effect on the expression of the pro-arrest gene p21. Similarly, acetylation of lysine 120 after DNA damage by the MYST family of acetyl transferases augments the pro-apoptotic function of p53 [152, 153]. Both acetylation and ubiquitylation at lysine 320 have been reported and they can

trigger apoptosis or growth arrest, respectively [154]. The ASPP family of proteins (apoptosis stimulating protein of p53) can bind and regulate the activities of p53 [155]. Chromatin immunoprecipitation (ChIP) assays revealed that binding of ASPP1 and ASPP2 to p53 can promote p53 interaction to promoters of pro-apoptotic genes, such as Bax and PIG3, but not to the cell cycle arrest gene CDKN1A or MDM2. Contrarily, iASSP binds p53 and inhibits p53-mediated apoptosis [156, 157]. Other proteins that are involved in the modulation of p53 target selectivity are the Brn3 family of POU domain transcription factors [158], the YB1 protein [159], and Hzf (hematopoietic zinc finger), a potent pro-survival regulator of p53 transactivation [160, 161]. The cellular apoptosis susceptibility gene hCAS/CSE1L plays a role in p53 promoter selection by enhancing the transcription of pro-apototic genes through gene-specific chromatin modifications. hCAS/CSE1L associates with PIG3 and p53AIP1 promoters (but not p21) and decreases H3K27me3 thus enhancing their transcription [162].

1.8.2 Regulation of p53 at RNA level

p53 mRNA is targeted for both translational and stability regulation by a variety of RNA-binding protein. For instance, PTB, hnRNPC1/C2, MDM2, RPL26, DAP5, Annexin A2, HuR and PSF bind to p53 mRNA and positively regulate its translation [163-169], while Pdcd4, RNPC1 and nucleolin negatively regulate p53 mRNA translation [166, 170, 171]. p53 protein itself can bind to its own 5′-UTR and inhibit its translation [172]. HuR protein can also bind to p53 3'UTR and increases p53 mRNA stability [173]. Another mechanism of 3′UTR mediated p53 regulation is through microRNAs. miR-125b, miR-504, miR-25, and miR-30d directly target the 3′UTR of p53 mRNA and down-regulate p53 protein levels, which results in reduced expression of genes that are transcriptionally activated by p53 [174].

1.9 THE N-MYC ONCOGENE

N-Myc is a transcription factor and a member of the MYC family of proto-oncogenes [175]. N-Myc regulates the expression of genes involved in proliferation, growth, apoptosis, energy metabolism, and differentiation and plays an essential role in normal brain development. N-Myc is expressed primarily in nervous tissues early during embryonic development, and is absent in most adult tissues. Amplification of N-Myc occurs in 20–22% neuroblastomas at diagnosis and 40% progressive neuroblastomas, an embryonic tumor derived from primitive cells of the sympathetic nervous system [176, 177]. N-Myc amplification has also been reported in retinoblastoma [178] and small cell lung carcinoma [179]. N-MYC amplification results in increased levels of cellular proliferation, decreased apoptosis, lack of cell differentiation and enhanced tumor vascularization [180]. Malignant neuroblastoma is one of

the most common and deadly solid tumor in infants and children and N-Myc amplification is the single most predictive factor for bad prognosis in this disease [181]. N-Myc knockdown by siRNA in neuroblastoma cells carrying N-Myc amplification can induce differentiation [182], and antisense strategies that target N-Myc inhibit mouse neuroblastoma tumorigenesis in vivo [183].

1.10 FAS

FAS (also referred as Apo1/CD95 receptor) is a member of the tumor necrosis factor (TNF) receptor family of transmembrane receptors. FAS receptor is expressed in almost all tissues, while its ligand FASL expression is restricted to activated lymphocytes and immune-privileged sites such as lung, brain, testis and eye [184]. In these immune-privileged sites, the expression of FASL leads to apoptosis and immunosuppression, providing protection from destructive inflammatory responses [185]. Binding of FASL to FAS receptor triggers recruitment of specific adaptor proteins, activation of caspase cascades and induction of apoptosis [186]. The recognition of FASL by FAS induces FAS trimerization and interaction of the adaptor FAS-associated death domain-containing protein (FADD) to the intracellular FAS death domain (DD). This in turn leads to recruitment, dimerization and activation of caspase-8 [187, 188]. Activated caspase-8 induces apoptosis either through direct cleavage and activation of caspase-3 [189], or through cleavage of Bid, a pro-apoptotic Bcl-2 family protein. Truncated Bid (tBid) translocates to mitochondria, inducing cytochrome c release, which sequentially activates caspase-9 and -3 [190].

FAS receptor gene is located on chromosome 10 in human cells and seven distinct protein isoforms have been identified and arise from alternative splicing of FAS receptor RNA. Only isoform 1 encodes the functional, full-length protein of 335 amino acids. It contains three cysteine-rich pseudo-repeats, a transmembrane domain and an intracellular death domain. Isoforms 2, 4, 5, 6 and 7 lack the transmembrane domain and are therefore soluble isoforms who are thought to sequester and inactivate FAS ligand thus antagonizing FAS-receptor-mediated apoptosis [191]. Isoform 3 does not contain a functional intracellular death domain, and it might be involved in resistance of fetal thymocytes to apoptosis following FAS receptor aggregation [192]. The soluble anti-apoptotic FAS isoforms are generated by skipping of exon 6 [193]. A number of splicing regulators have been found to influence this splicing event including PTB, TIA1, HuR, hnRNPC, EWS and RBM5 [194-200].

1.11 POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Regulation of gene expression is a fundamental process through which mammalian cells regulate cell survival, adaptation to stress, homeostasis, cell fate and differentiation, in response to different stimuli and environmental signals [201]. Newly transcribed mRNAs are subjected to posttranscriptional events that dictate the levels of the encoded proteins. These events comprise mRNA processing, nucleo-cytoplasmic export, mRNA localization, mRNA stabilization and translational regulation. All these processes involve the binding of proteins (trans-acting factors) to regulatory sequences on coding or untranslated regions of mRNAs (cis-acting elements). There are several types of cis-acting elements that affect the fate of the mRNA and they can be distributed throughout the transcript sequence.

The 5′ UTRs can contain:

- upstream open reading frame (uORF), it can affect the efficiency of translation initiation of the main downstream ORF or trigger mRNA decay [202];
- internal ribosome entry site (IREs), it promotes translation initiation independently of the presence of the commonly utilized 5'-7mG cap [203];
- iron responsive element (IRE), secondary stem-loop structure of 26–30 nucleotides found in the mRNA of proteins involved in iron metabolism, it regulates their translation or stability [204];
- 5′ terminal oligopyrimidine tract (5′TOP), it contains a cytidine residue at the cap site followed by a stretch of up to 13 pyrimidines and it is critical for translational control all of which are known to be involved in translation regulation [205];
- polypyrimidine tract, a 15-20 base pairs long pyrimidine rich region located about 5-40 base pairs before the 3' end of introns, it affects early stages of the splicing process [206];
- miRNAs, small noncoding RNAs that promotes degradation or translation repression of the target mRNA through sequence complementarity [207, 208].

On the coding region:

- the coding region determinant (CRD), a non-sequence specific element, about 80-90 nt long, that affects both mRNA stability and translation [209, 210];
- miRNAs, see above [211-213].

The 3′ UTRs contain cis elements such as:

- zipcode sequences, highly variable in length, structure and complexity, they can regulate mRNA localization, anchorage and translation [214, 215];

- AU-rich elements (ARE), see following paragraph [216];
- GU-rich element (GRE), a highly conserved sequence (UGU[G/U]UGU[G/U]UGU) that targets mRNA for rapid deadenylation and degradation [217, 218];
- CA-rich elements (CAREs), CA-dinucleotide repeats exerting stabilization effects on mRNA [219];
- miRNAs, see above [220];
- poly(A) signals (PASs), consensus AAUAAA sequences located about 10-35 nucleotides upstream of the actual site of poly(A) tail addition [221, 222].

The length of the 3′ UTR is determined by the maturation process of the 3′ end of the pre-mRNA, which results in cleavage and polyadenylation of the mRNA. Alternative polyadenylation in the 3′UTR generates different mRNA isoforms containing distinctive cis-acting elements, thus conferring different stabilities and/or translation properties to the corresponding mRNAs [223].

1.11.1 AU-rich elements

Described for the first time in 1986 in the 3'-UTR of unstable mRNAs coding for cytokines [224], adenylate/uridylate-rich elements (AU‐rich elements or AREs) are now the most well studied group of cis-acting elements. AREs are found in the 3'UTR of short-lived mRNAs, typically proto-oncogene and inflammatory mediators, and are important in controlling mRNA stability and translation. The basic core of the canonical ARE consists of an AUUUA pentamer embedded in an AU-rich context. Based on the number and the distribution of AUUUA pentamers, AREs have been divided into three classes [216, 225]. Class I AREs are characterized by several scattered AUUUA motif within U-rich regions (e.g. AREs found in c-myc and c-fos). Class II AREs contain at least 2 or more overlapping UUAUUUA(U/A)(U/A) nonamers (e.g. AREs found in TNF alpha and VEGF). Class III AREs are much less well defined, they lack the AUUUA motif but contain a U-rich regions (e.g. AREs found in c-jun and p53). Bioinformatics evaluations estimate that 5–8% of human genes contain ARE sequences [226].

1.11.2 AU-rich element binding proteins

AU-rich elements are bound by a set of RNA-binding factors collectively called ARE-binding proteins (ARE-BPs). Over twenty ARE-BPs have been identified until now [227, 228] and their RNA-binding domains include RNA Recognition Motifs (RRMs), zinc fingers and K homology (KH)-domains. Some ARE-BPs can promote mRNA degradation, for example tristetraprolin (TTP) [229, 230], butyrate-regulated factor-1 (BRF1) [230] and KH domain-splicing regulatory protein (KSRP) [231]; others work as mRNA stabilizers, for example human antigen R (HuR) [232] and the poly(A)-binding protein-interacting protein 2 (PAIP2) [233]; some can do both, for example AUF1/hnRNP D [228]. Yet another subset of ARE-BPs represses translation of their mRNA targets, for example the T-cell intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) [234]. Both cooperation and antagonism between different ARE-BPs has been observed in regulation of mRNA expression of a common target [235, 236]. Furthermore, subcellular localization of the targeted transcript and/or post-translational modifications of ARE-BPs are also important factors that determine the final outcome of the regulation [237, 238].

1.11.3 AU-rich element mediated mRNA decay (AMD)

The mRNA levels inside a cell depend on the balance between transcription and degradation rates. Transcripts that contain AREs are particularly susceptible to mRNA decay through a process known as ARE-mediated decay (AMD) [239]. AMD can promote mRNA degradation by three major pathways: 1) shortening of the poly-A tail (deadenylation) followed by 3'-to-5' mRNA degradation via the exosome; 2) deadenylation followed by removal of the 5′ cap (decapping) and 5'-to-3' mRNA decay; 3) miRNA-mediated endoribonucleolytic cleavage. Most ARE-BPs are not the direct executors of AMD, instead they recruit and regulate enzymes of the RNA degradation machinery.

Deadenylation is the first and often rate-limiting step in the regulated mRNA decay pathways. Two cytoplasmic deadenylase complexes (Pan2/Pan3) contribute to the reduction in poly(A) tails, followed by a large complex of exoribonucleases and adaptor proteins termed the Ccr4–Not complex that hydrolyze the poly(A) tail progressively reducing its length to 10-15 As. After deadenylation, the unprotected 3′-end of the mRNA is degraded by the exosome, a protein complex (10-12 subunits) consisting of 3′-to-5′ exo- and endoribonucleases [240].

Deadenylation also triggers 5′-to-3′ RNA decay, starting from the removal of the 5′-m^7G-cap by the Dcp1/Dcp2 decapping complex [241] and followed by 5′-to-3′ degradation promoted by the cytoplasmic exonuclease Xrn1 [242, 243]. Decapping of the 5′- m^7G -cap structure is a

prerequisite for 5′–3′ decay since Xrn1 has less than 1% relative activity on capped RNAs [244].

The third AMD decay pathway involves the mediation of microRNA. MicroRNAs (miRNAs) are short RNA molecules (20-22 nts) involved in post-transcriptional gene regulation. miRNAs associate with a protein complex termed the RNA-induced silencing complex (RISC). The RISC complex can target RNAs that show complementarity to the miRNA, leading to site-specific endonuclease cleavage by Ago2 (a component of the RISC) and degradation of the resulting target RNA by Xrn1 and the exosome. miRNAs can bind to AREs and enhance or inhibit the action of ARE-BPs to regulate ARE bearing transcripts [239, 245-248]. However, a recent study showed that AMD can function independently of miRNAs in mouse and Drosophila cells [249].

Cytoplasmic mRNA localization can also be affected by the presence of AU-rich elements on their 3'UTRs. Processing bodies (P-bodies or PBs) are distinct cytoplasmic granules that contain components of the mRNA degradation machinery necessary for 5'-to-3' mRNA decay [250]. Decay promoting ARE-BPs, such as TTP and BRF-1/2, are found in P-bodies, suggesting that they may recruit ARE-containing mRNAs to P-bodies for degradation [251]. Differently, stress granules (SGs) are a separate type of cytoplasmic granule that can be induced upon cellular stress. Transcripts in SGs are subjected to mRNA triage and, depending on the duration and severity of the stress, re-routed to sites of translation reinitiation, storage, or to P-bodies for decay [252]. The ARE-BPs TIA-1, TIAR, and TTP among others, are components of stress granules and recruit their target transcripts to stress granules in an ARE-dependent manner. A comprehensive review by von Roretz et al. discusses the regulation of ARE-containing mRNAs by ARE-BPs in response to stress, along with the involvement of cytoplasmic granules [253].

1.11.4 Importance of RNA 2D and 3D structure

RNA is a polymer composed by the combination of the A, U, C and G ribonucleotides. RNA is a single-stranded molecule and complementary sections within a single strand of RNA can base-pair with each other, causing the molecule to fold itself and form a complex, three-dimensional shape. RNA can form A–U and G–C base pairing, just like DNA, but the non-Watson–Crick G–U wobble pair is also commonly observed and has approximately the same stability as an A–U [254]. RNA 2D and 3D architectures are critical for many aspects of cellular physiology such as RNA transcription, splicing, translation, localization and turnover [255-259]. Moreover, RNA molecules not only carry the DNA-encoded information from the nucleus to the cytoplasm, but they also catalyze biochemical reactions as exemplified by ribozymes [260]. Unlike DNA binding proteins, which typically recognize features in the major groove of double-stranded DNA, RNA binding proteins, through their RNA binding

domain, recognize single-stranded RNA, double-stranded RNA, 3D structural features of folded RNAs or they may bind RNA non-specifically [261].

In the last few decades, RNA structure prediction has been the focus of much research aiming at developing tools and computational methodologies for the analysis of RNA sequences. The aim is to identify different RNA secondary and tertiary structures, understand their biological functions and how these structures can affect protein/RNA interactions. RNA bioinformatics provides tools for folding of single and multiple RNA sequences, thus contributing in the description of RNA–RNA interactions as well as RNA–protein interactions.

The most commonly applied 2D structure prediction methods rely on folding thermodynamics [262], but there are complementary approaches that use, for example, kinetics or probabilistic models [263, 264]. 3D structure prediction is a developing frontier in RNA biology. While there are a number of algorithms able to predict correct 3D structure of smaller RNA molecules, the problems appear with the prediction of known complex structures, thus, the question whether these tools are accurate and reliable for the prediction of new structures. Available methods for RNA 3D structure prediction and experimental techniques that provide RNA structural information are reviewed in [265].

1.12 PROTEIN-RNA INTERACTION ANALYSIS AND HIGH-THROUGHPUT TECHNIQUES

Identification of RNA–protein interactions rely on the combination of both experimental and computational methods. Experimental methods include RNA co-immunoprecipitation from cell or tissue extracts to determine RNA targets (RIP). In the earlier approaches bound RNA was identified using microarrays (RIP-chip) [266] or more recently by RNA sequencing (RIP-Seq) [267]. However, RIP–chip and RIP-Seq capture only the relatively stable protein–RNA complexes, requiring the application of complementary methods to detect the more transient interactions. UV cross-linking and immunoprecipitation and sequencing (CLIP-Seq) was then developed (also called HITS-CLIP for High-throughput sequencing of RNA isolated by cross-linking and immunoprecipitation) [268]. CLIP exploits the photoreactivity of pyrimidines and specific amino acids to create covalent crosslinks between RNA binding proteins and their interacting RNAs and allows to identify the site of the interaction at a good resolution. Interestingly, HITS-CLIP was also applied to Argonaute (Ago)/miRNA complexes, providing a molecular view on miRNA–mRNA interactions [269]. Another variant includes PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation), which differs from CLIP as it features the incorporation of the 4-thiouridine (4-SU) and 6-thioguanosine (6-SG) photoreactive nucleotide analogs into RNA during transcription [270]. This modification increases the efficiency of the UV crosslinking and the accuracy in mapping the binding site. An additional improvement is represented by iCLIP (individual-nucleotide resolution CLIP), where efficiency of CLIP is further optimized

thanks to a different cDNA cloning protocol and circularization of the reverse transcription products, which enables identification of the cDNAs that truncate at the cross-link sites [271]. An extensive review by Milek et al. gathers novel applications of high-throughput sequencing to study protein–RNA interactions [272].

2 AIM OF THIS THESIS

The overall aim of this thesis was to characterize the Wig-1 protein function and to identify its associated RNA targets. Moreover, we aimed to investigate the biological implication of the regulation of some of these targets.

The specific aims of each paper were:

Paper I: To study the mechanism of Wig-1-mediated regulation of p53 mRNA.

Paper II: To study the mechanism of Wig-1-mediated regulation of N-Myc mRNA and the implication in neuroblastoma cancer cells differentiation.

Paper III: To study the effects of Wig-1 knockdown on global gene expression and cell survival with focus on the mechanism of Wig-1-mediated regulation of the pro-apoptotic FAS at the RNA level.

Paper IV: To characterize the RNA-binding properties of Wig-1 and identify the Wig-1 interacting transcriptome.

3 RESULTS AND DISCUSSION

3.1 PAPER I

The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element

Wig-1 is a p53 transcriptional target and with the intent to elucidate Wig-1 biological function we were intrigued by the observation that Wig-1 knockdown using siRNA led to decreased p53 protein levels. We were then able to confirm that Wig-1 silencing causes p53 protein levels reduction both in unstressed cells and after p53 activation with the DNA-damaging drugs. Additionally, overexpression of exogenous Wig-1 not targetable by siRNA rescued p53 levels, demonstrating that the decrease on p53 levels is a consequence of endogenous Wig-1 loss. Moreover, we could show that Wig-1 had a positive role in the p53 response to cellular stress.

Wig-1 is an RNA-binding protein [15, 16] and our lab have previously described the interaction between Wig-1 protein and two RNA-binding proteins involved in all aspects of RNA regulation, i.e. RHA and hnRNPA2/B1 [17]. We therefore asked if Wig-1 was regulating p53 RNA levels as well. This was indeed the case, in fact, we showed that absence of Wig-1 led to increased p53 mRNA decay and increased p53 mRNA deadenylation. Localization studies indicated that Wig-1 protein is present in both nucleus and cytoplasm, and more importantly it was found to be able to shuttle from nucleus to cytoplasm, the compartment where mRNA decay takes place [238, 256].

In order to understand which region in p53 mRNA was responsible for the regulation, we tested different deletion constructs of the p53 transcript. Through GUS reported assay we verified that the U-rich region in p53 3'UTR is responsible for Wig-1-mediated regulation of p53. The same region was also found to be crucial for Wig-1 binding to p53 mRNA as assessed by biotin pulldown assay. Moreover, we confirmed binding of Wig-1 to endogenous p53 mRNA by RNA immunoprecipitation assay.

In summary, the main findings of this paper are:

- Wig-1 binds and stabilized p53 mRNA through a U-rich element on p53 3'UTR
- Wig-1-p53 positive feedback loop is important for maintaining the basal p53 mRNA levels and to augment the p53 response to cellular stress.

3.2 PAPER II

Wig-1, a novel regulator of N-Myc mRNA and N-Myc-driven tumor growth

In paper I we published that Wig-1 binds and regulates p53 mRNA through a U-rich element on the 3'UTR. U-rich elements belong to the family of AU-rich elements (ARE). Thanks to this finding we could place Wig-1 in the group of the AU-rich element binding proteins (ARE-BPs). Many well-studied ARE-BPs can regulate a number of different targets [228]. In order to find novel Wig-1 regulated transcripts, we knocked down Wig-1 and tested the levels of a number of proteins whose mRNA was previously reported to be targeted by ARE-BPs. N-Myc protein, whose mRNA has been previously reported to be stabilized by the ARE-BP HuD [273, 274], was included in the screen and found to be dramatically reduced in the neuroblastoma cell line SK-N-BE(2) after Wig-1 silencing. This discovery set the stage for what became paper II.

We started by confirming our preliminary data by knocking down Wig-1 in two neuroblastoma cell lines carrying N-Myc amplification, SK-N-BE(2) and Kelly. We detected a significant reduction in both N-Myc protein and mRNA levels. We next investigated what region in N-Myc mRNA is required for Wig-1-mediated regulation. Looking at N-Myc 3'UTR we identified a U-rich element and a general AU-rich region containing two canonical AREs (referred as proximal and distal ARE). We generated different deletion constructs that we tested through a luciferase reporter assay and a pulldown assay and we found that the proximal ARE was determining factor for the binding and the regulation.

We then wanted to investigate if Wig-1 mediated regulation of N-Myc mRNA had any relevant biological consequences in neuroblastoma cells. It has been previously reported that N-Myc knockdown causes differentiation in SK-N-BE(2) cells carrying amplified N-Myc [182]. We knocked down Wig-1 in these cells and looked for differentiation marker and changes in morphology. We recorded substantial morphological changes, increased cell cycle arrest in G1 phase and upregulation of the differentiation marker Neuropeptide Y (NPY) already 4 days after Wig-1 silencing, suggesting that inhibition of N-Myc expression resulted in differentiation. Overexpression of exogenous N-Myc lacking the 3'UTR (thus lacking the region responsible for Wig-1 mediated regulation) could rescue cells from undergoing differentiation, proving that the differentiated phenotype observed after Wig-1 knockdown is a direct consequence of Wig-1 effect on N-Myc mRNA. Furthermore, as we could not detect any signs for differentiation after Wig-1 knockdown in SH-SY5Y neuroblastoma cells that do not carry amplified N-Myc, we conclude that the effect of Wig-1 knockdown on differentiation of cells carrying amplified N-Myc is due to attenuated N-Myc expression.

We next investigated if Wig-1 knockdown had any impact on tumor formation *in vivo*. We inoculated SK-N-BE(2) cells pre-transfected with siRNA against Wig-1 or control siRNA

subcutaneously in nude mice. Tumor take in the xenograft model was delayed in SK-N-BE(2) cells pre-transfected with siRNA targeting Wig-1 (with a mean of 24 days) compared with control siRNA (with a mean of 19.5 days). Thus, transient Wig-1 knockdown is sufficient to significantly delay development of N-Myc-driven tumors in nude mice.

The findings included in this study suggest that the design and the development of a therapeutic strategy to target either Wig-1 expression or Wig-1 protein/N-Myc mRNA interaction could be an alternative or an improvement to current therapeutic strategies in the treatment of aggressive and recurrent neuroblastoma carrying amplified N-Myc, which is the most deadly cancers in children [275].

Moreover, we also showed that treatment of IMR-32 neuroblastoma cells, which carry wild-type p53, with chemotherapeutic drug led to activation of p53 and increased levels of both Wig-1 and N-Myc protein. This suggests the idea of a p53-Wig-1-N-Myc axis that is activated upon stress. According to this model, activated p53 will induce cell cycle arrest genes and Wig-1; Wig-1 will then stabilize N-Myc and promote the recovery and cell survival after p53-induced cell cycle arrest in response to stress. Meanwhile, Wig-1-mediated stabilization of p53 mRNA will guarantee basal levels of p53 and prompt action in case of persistent stress.

To summarize, the main findings of this paper are:

- Wig-1 as a novel regulator of N-Myc mRNA.
- Wig-1 binds and stabilizes N-Myc mRNA through an ARE-containing region in the N-Myc 3'UTR.
- Loss of Wig-1 in SK-N-BE(2) neuroblastoma cells carrying amplified N-Myc triggers cell differentiation as a consequence of Wig-1-mediated regulation of N-Myc mRNA.
- Wig-1 knockdown is sufficient to significantly delay development of N-Myc-driven tumors in mice.

3.3 PAPER III

Wig-1 regulates cell cycle arrest and cell death through the p53 targets FAS and 14-3-3σ

Papers I and II describe Wig-1-mediated regulation of the tumor suppressor p53 and the N-Myc oncogene at RNA level through two distinct AU-rich elements in their 3'UTR. Wig-1 is undeniably involved in post-transcriptional gene regulation and to gain an overall picture of the effect of Wig-1 on global gene expression we decided to perform microarray analysis after Wig-1 knockdown in the HCT116 colon carcinoma cell line. Loss of Wig-1 led to deregulation by more than four-fold of 2447 transcripts that are involved in pathways such as

Alzheimer's and Huntington's diseases, p53 pathway, FAS signaling pathway and apoptosis. We showed in Paper I that the p53 target Wig-1 is a regulator of p53 mRNA, thus it wasn't surprising to find the p53 pathway and the apoptosis pathway among most significantly affected pathways. We then moved on to validate our microarray data; six out of eight of the targets chosen for validation were confirmed at protein level (i.e. FAS, WNT1, AKT3, APP, 14-3-3σ and PPP2CB). We found that Wig-1 silencing led to increase levels of the proapoptotic factor FAS and reduced levels of the cell cycle arrest associated factor 14-3-3σ, suggesting that Wig-1 can modulate the cellular stress response such that cell cycle arrest is favored over apoptosis. Through FACS-PI analysis and apoptotic assays, we confirmed that Wig-1 silencing enhanced apoptosis and reduced cell cycle arrest in response to cellular stress in HCT116 cells. Notably, our data demonstrated that Wig-1 regulates FAS and 14-3-3σ independently of p53, as evidenced by the comparable effect in both p53 wt and null HCT116 cells with and without stress. We suggested that Wig-1 acts to maintain high levels of 14-3-3σ (thus promoting cell cycle arrest) while at the same time inhibiting FAS (and thus, repressing apoptosis), the net outcome being increased survival.

We found that Wig-1 binds to the ARE in the 3'-UTR of FAS mRNA and that the binding is mediated by the first zinc-finger in Wig-1. Moreover, this ARE is responsible for Wig-1-mediated regulation of FAS mRNA and its removal completely abrogates binding and regulation.

We found that Wig-1 co-localizes and interacts with a component of the deadenylase complex CCR4–NOT, CNOT6 [276], suggesting that Wig-1 may enhance FAS mRNA degradation by enabling the interaction between FAS mRNA and the deadenylase complex. Supporting this hypothesis, we found Wig-1 or CNOT6 knockdown results in comparable increases in FAS mRNA levels. We conclude that FAS mRNA regulation is associated with altered deadenylation rate mediated by the CCR4–NOT complex.

We could also demonstrate that Wig-1, CNOT6 and FAS mRNA localize simultaneously inside stress granules (SGs), cytoplasmic granules important in regulation of mRNA turnover upon stress-induced translational arrest [277]. We suggest that the co-localization of Wig-1, CNOT6 and FAS mRNA inside SGs facilitates the interaction between these three factors, enhancing FAS mRNA degradation and/or re-localization to other specialized mRNA degradation sites.

The findings presented in this paper provide a better understanding of the biological function of Wig-1 protein and propose a role of Wig-1 in the AU-rich mediated decay via the 3'-to-5' RNA decay pathway. Moreover we describe here that the Wig-1 ARE-BP in addition to being an RNA stabilizer (p53 and N-Myc) can also act as a destabilizer (FAS), in line with what reported for other ARE-BPs such as AUF1 and HuR [278-281].

Additionally, this is the first report that shows regulation of FAS mRNA stability at post-transcriptional level.

In summary, the main findings of this paper are:

- Wig-1 silencing affects a large set of mRNAs directly or indirectly
- Wig-1 acts as a survival factor promoting arrest rather than apoptosis
- Wig-1 binds and destabilizes FAS mRNA through an ARE on its 3'UTR
- Wig-1 interacts with the deadenylase complex CCR4-NOT and FAS mRNA in the cytoplasmic stress granules

3.4 PAPER IV

Genome-wide identification of Wig-1 mRNA targets by RIP-Seq analysis

In paper III we determined a list of mRNAs whose expression was affected by Wig-1, directly or indirectly. Another missing piece of the puzzle crucial in order to build an accurate model of cellular RNA-Wig-1 network was to probe the entire Wig-1-bound mRNA repertoire. In paper IV we have performed a systematic analysis of Wig-1-associated mRNAs, taking advantage from modern large-scale technologies. Through RNA immunoprecipitation followed by high-throughput sequencing (RIP-Seq) in HCT116 and Saos-2 cells, we found 286 Wig-1-bound mRNAs common in the two cell lines tested (HCT116 and Saos-2). In agreement with what we found in Paper III, FAS mRNA was found enriched in HCT116 cells. Network Enrichment Analysis indicated that Wig-1 targets are highly connected with the Cell Cycle pathways and is in accordance with what we described in Papers II and III.

In order to validate the list of Wig-1-bound mRNAs, we selected nine Wig-1 associated mRNAs enriched in both HCT116 and Saos2 RIP-Seq experiments. We could validate 100% of the targets in the HCT116 cell line (CCNG1, RMI1, CHEK1, MTHFD2, CAV1, AMD1, HIF1A, MAD2L1 and EIF4E) and 67% of the targets in Saos-2 (MAD2L1, MTHFD2, RMI1, EIF4E, CHEK1 and AMD1). The difference in validation efficiency in the two cell lines can be explained by greater variation between replicates observed for Saos-2 as compared to HCT116, which generates large standard deviation and decrease the significance of the data. Despite this, we can appreciate in all selected targets a tendency that is concordant with what observed in the RIP-Seq results. Additionally, we found that Wig-1 knockdown in HCT116 cells led to decreased levels of MTHFD2, EIF4E, RMI1, and CAV1 mRNA while we observed an increase in the levels of HIF1A mRNA, all of which are important factors in tumor biology. These data underlines the role of Wig-1 in the regulation of cell cycle and cell proliferation, tumor onset, progression and metastasis. Our data

confirms that Wig-1 can both stabilize or destabilizes its RNA targets, including pro and anti-proliferation factors.

Our study helped also to further characterize Wig-1 mRNA-binding properties. Wig-1 preferentially binds to mRNAs that carry AREs in their 3'UTRs as confirmed by the fact that 95% of its bound targets mRNAs contain at least one AUUUA pentamer. Furthermore, de novo motif enrichment analysis revealed that Wig-1 favors the binding of motifs that are generally rich in A and Us compared to unbound control mRNAs.

Both primary and secondary RNA structure elements are involved in describing protein/RNA interaction, therefore we decided to analyze whether the 3'UTR sequences of Wig-1-bound RNAs share a consensus secondary structures using the LocARNA software. We obtained a common consensus 2D motif shared among the nine validated targets that is characterized by very low GC content. Moreover the shared 2D motif is frequently situated in close proximity to an AU-rich element in the primary sequence of the 3'UTR or in the secondary folded structure on the 3'UTR. However, experimental validation of *in silico* predictions of structural elements is necessary since many factors other than nucleotides sequence can influence in vivo structure (e.g. binding of co-factors and solvent properties).

Our work provides a comprehensive view of the RNA-binding properties of Wig-1 and helps to better define the Wig-1-RNA interacting network. Our data confirm that Wig-1 is an ARE-BP involved in regulation of cell cycle progression and cell proliferation. Moreover, we significantly expand the list of known Wig-1 targets, and provide additional insights into preferred Wig-1 RNA binding motifs.

To summarize, the main findings of this paper are:

- Wig-1 binds more than two hundred mRNAs in HCT116 and Saos-2 cells
- Wig-1 mRNA targets are functionally enriched in the cell cycle pathway
- Wig-1 preferentially binds to mRNAs that carry AREs in their 3'UTRs, thus corroborating Wig-1 as an AU-rich element binding protein
- Wig-1 bound mRNAs share a consensus 2D motif that might be important for Wig-1 interaction.

4 CONCLUSION AND FINAL DIRECTIONS

I have been asked several times if Wig-1 is a good or bad guy. There is no simple answer to this question. The Wig-1 gene maps in a chromosomal area that is commonly amplified in cancer and its expression levels has also been found elevated in many tumor samples (Figure 5 and [83]). This makes us believe that Wig-1 is behaving more as an oncogene than a tumor suppressor. Moreover, we show in this thesis that Wig-1 is stabilizing the mRNA levels of oncogenes such as N-Myc and possibly others like MAD2L1, MTHFD2 and EIF4E. But this is only part of the story. We believe that Wig-1 is a pro-survival factor and that it plays an important role in different processes depending on the cellular context.

Wig-1 is a transcriptional target of the p53 tumor suppressor gene [5]. Its level increases after p53 activation and has a p53 response element on the promoter that resembles those of pro-arrest p53 target genes (Figure 1) [45]. It has a defined role within the p53 pathway, which is to stabilize p53 mRNA levels and therefore to potentiate the p53 response to stress. At the same time Wig-1 directly regulates downstream targets of p53, such as 14-3-3σ and FAS. Wig-1 promotes cell cycle arrest through induction of 14-3-3σ whereas it reduces cell death promoting the decay of the pro-apoptotic FAS receptor. Moreover, a recent study showed that Wig-1 prevents senescence by promoting miRNA-mediated decay of the p53 target p21 [282]. Thus, the induction of cell cycle arrest by Wig-1 may serve as a temporary pause during which cells can repair the damage and limit cell death. Concurrently, to prevent cells to undergo a terminal proliferation arrest, i.e. senescence, Wig-1 affects p21 decay. Additionally, Wig-1 may affect other downstream effects of the p53-dependent stress response by regulating additional p53 targets that we haven't investigated or identified yet. For instance, we understand from Paper IV that Wig-1 binds and regulates HIF1A mRNA levels. HIF1A is a transcription factor that has an essential role in cellular and systemic responses to hypoxia and from previous publications we know that p53 negatively regulates both HIF1A transcriptional activity and HIF1A protein levels [283, 284]; furthermore, our data suggests that p53 might regulate HIF1A expression through its transcriptional target Wig-1. Therefore it would be interesting to examine if Wig-1 plays also a role in the regulation of hypoxia-induced p53-dependent apoptosis.

Besides the key role of Wig-1 in modulating the p53 response to stress through regulation of p53 itself and components of the p53 pathway, we believe that Wig-1 carries out important functions in other critical cellular processes, specifically stem cell maintenance and differentiation [12]. Wig-1 is essential for cell proliferation and the effort to generate a Wig-1 knockout mouse ended up giving some headaches, since we found Wig-1 deletion to be lethal as early as before blastocyst stage (unpublished results). Wig-1 expression levels decreases in cells that undergo differentiation as compared to the levels of their stem cells progenitors [106]. We also showed in Paper II that Wig-1 knockdown induces differentiation in neuroblastoma cells, and that in this particular case, this is attributable to Wig-1 mediated

regulation of N-Myc. Wig-1 expression is also regulated by a number of factors involved in differentiation and stem cell maintenance, such as the NGF, SPI1, ETS1, ELF1, MAX, BMI-1 and p53 [107-112]. Intriguingly, Wig-1 promoter in mouse embryonic stem cells has been found to be a so called "bivalent promoter" meaning that it simultaneously bears both the activating (H3K4me3) and the repressive (H3K27me3) marks [26, 29]. These bivalent domains were first found in pluripotent embryonic stem cells and are considered to be a feature of developmentally regulated genes [285]. These genes are maintained repressed until the time when a particular differentiation signal will switch the activating mark on and engage the cell to develop into a more specific functional type. Following differentiation, bivalent promoters embrace either an active or a repressed state, depending on cell fate. Interestingly, our observation that Wig-1 level decreases in late passages primary fibroblast as compared to early-passage fibroblast suggests that Wig-1 might be downregulated in replicative senescence and perhaps chromatin repression is a trigger. Thus, epigenetic regulation of Wig-1 may affect its function depending on cell type, developmental stage and presence or absence of specific stimuli, making Wig-1 a very versatile, dynamic and context-dependent effector.

We have shown that Wig-1 has numerous targets that are involved in different pathways. Possibly, the above mentioned chromatin modification might also influence Wig-1 target selectivity and the outcome of the regulation of a specific mRNAs. Moreover, post-translational modification (PTM) may also affect Wig-1 function, localization and stability as it is has been reported for a number of other ARE-BPs [253]. In our lab we are currently mapping Wig-1 PTMs and interesting data have been produced. Particularly, a number of modified sites are present exclusively in one of the two known Wig-1 species, suggesting that these different species might have different functions or properties (Xu et al., unpublished results). Additionally, some modified amino acids specifically located in the zinc finger domains, i.e. Wig-1 RNA binding motifs, are likely to be crucial for Wig-1 RNA-binding ability and might be affecting Wig-1 target selectivity. Further investigation will tell us which PTMs are central for Wig-1 function, under what circumstances and in what way they affect Wig-1 localization, stability, and more generally Wig-1-mediated post-transcriptional regulatory properties.

The example of well-studied ARE-BPs denotes that protein-protein interaction is also relevant when deciding target choice and fate [286, 287]. In this thesis introduction I listed a number of protein that have been found to interact with Wig-1 (Table 1), although no studies have characterized the biological meaning of it, yet. Interestingly enough, we notice that several of these proteins are involved in mRNA processing, e.g. DICER1, SF3A1, hnRNPA2/B1 and TARBP2. Ahead of us, a good amount of lab work needs to be performed in order to obtain a clearer picture of Wig-1 protein interacting network. In paper IV we demonstrate Wig-1 interaction with CNOT6 protein [276], a component of the deadenylation complex, indicating a possible molecular mechanism for Wig-1-mediated ARE-dependent regulation.

Both Wig-1 overexpression and knockdown has a negative effect on cell viability, indicating the importance of monitoring and maintaining Wig-1 levels correctly balanced in the cell. This is most likely achieved through a well-coordinated regulatory system. Thanks to modern high-throughput methods, a lot of biological data are produced and shared everyday. Part of this information includes Wig-1 interaction with and/or regulation by transcription factors, miRNAs, and proteins involved in all aspect of gene regulation, from epigenetic to transcription, from mRNA turnover and translation to protein stability (Tables 1-4). The next challenge is to translate these data and generate biological models that would serve to gain further knowledge and understanding of the molecular basis behind relevant cellular processes, from tumor development to tumor progression, from embryonic development to senescence.

So, coming back to the initial question: Is Wig-1 a good or a bad guy? If you have been reading so far, you are probably a life scientist and you might find this question a bit odd. There are no bad guys inside a cell in my opinion. There are, though, events that happen and disturb the harmony. Wig-1 is involved in maintaining this harmony and it does that together with p53. But Wig-1 does much more, and that "more" is so important that there is no life without Wig-1.

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