Thesis for doctoral degree (Ph.D.)
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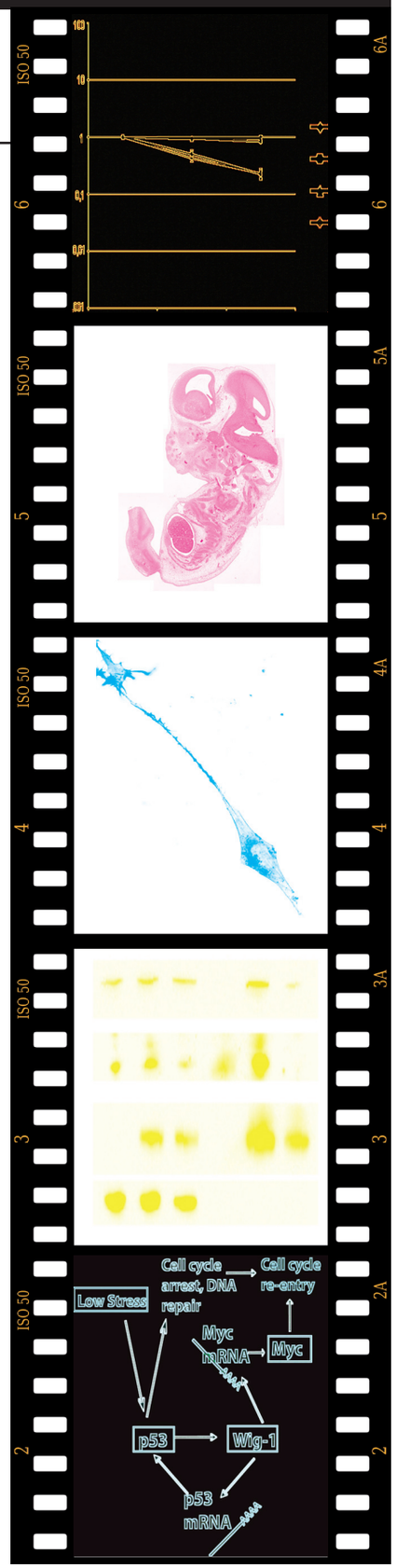
Wig-1: A p53 target that regulates the mRNA of p53 and Myc - and more?



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Wig-1: A p53 target that regulates the mRNA of p53 and Myc - and more?

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Cover image: “The negative of my thesis”. Thanks to Salah for inspiring the idea and to Marcus for carrying it out.

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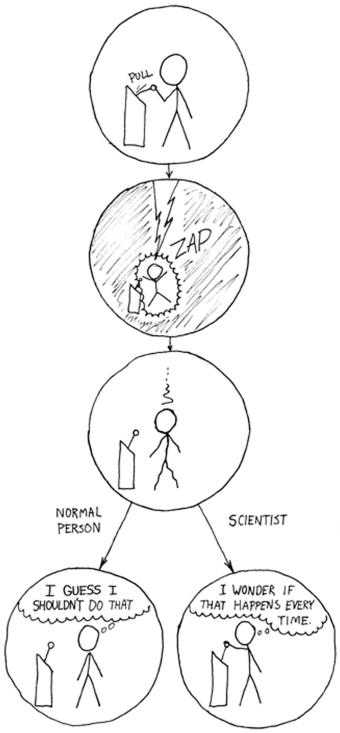
To Marcus

To my parents

"Nature was not designed to make life easy for biologists"
Colin Tudge



Adopted from xkcd.com

Abstract

Wig-1 is a transcriptional target of the tumor suppressor p53. p53 is activated by cellular stress and can induce a wide variety of responses. Some – like apoptosis – follow upon severe damage, while milder damage results in outcomes such as cell cycle arrest and DNA repair. Yet other p53 functions rely on physiological p53 levels. p53 mainly exerts its functions through inducing the transcription of target genes, hence in order to understand the function of p53 one must understand the function of these targets. Wig-1 was identified as a p53 target more than ten years ago, and was found to be a double stranded RNA binding protein. Apart from that, however, its function has remained elusive. In this work we demonstrate that Wig-1 regulates mRNA stability through binding to so-called AU-rich elements in 3'UTRs, and we identify p53 as well as N- and c-Myc as its targets. We also report that Wig-1 knockout causes early embryonic lethality in mice, probably due to dysregulation of Wig-1 targets such as Myc. Thus we show that p53, through Wig-1, can activate Myc and possibly other pro-survival targets. This activation may represent a way of facilitating for cells to recommence cycling after a repaired damage. Simultaneously, increased Wig-1 sensitizes the cell to any remaining damage by also stabilizing the p53 mRNA. In conclusion, we have found that the p53 target Wig-1 regulates mRNA stability through AU-rich elements. We propose a novel mechanism by which p53, through Wig-1, can tweak the cell milieu toward survival.

Populärvetenskaplig sammanfattning

I den här avhandlingen har vi beskrivit funktionen av Wig-1, en gen som aktiveras av den kända tumörsuppressorn p53. p53 hindrar celler från att förvandlas till cancerceller, framför allt genom att aktivera andra gener som sedan har olika anti-cancer-funktioner i cellen. Aktiverat p53 kan antingen leda till att cellen tar en paus och lagar eventuell skada (vilket ofta händer vid mildare stress) eller att den dör (om den är allvarigt skadad). För att riktigt förstå hur p53 fungerar är det viktigt att förstå funktionen av p53:s målgener, och därför har vi studerat funktionen av Wig-1. Vi har funnit att Wig-1 kan reglera stabiliteten av vissa s.k. mRNA i cellen, och därmed kan Wig-1 reglera nivåerna av de proteiner som kodas för i dessa mRNA. Eftersom det framförallt är proteiner som utför de olika funktionerna i cellen, kan Wig-1 påverka hur en cell reagerar på t.ex. stress. Vi har funnit att Wig-1 på detta sätt kan stabilisera p53, och dessutom stabilisera Myc, en gen som är känd för att driva på celltillväxt och för att inducera cancer. Fyndet att Wig-1 reglerar Myc antyder att Wig-1 kan bidra till att celler överlever och delar sig. Dessutom har vi funnit att möss som helt saknar Wig-1 dör väldigt tidigt under embryonalutvecklingen, förmodligen för att mRNA som i vanliga fall kontrolleras av Wig-1 inte längre regleras som de ska. Även dessa resultat visar på en viktig roll för Wig-1 i cell-överlevnad. Vi tror att Wig-1 kan fungera så att den påverkar balansen i p53:s reaktion på mildare stress i riktning mot ett svar som möjliggör att cellen kan ta en paus, reparera eventuell skada, och sedan fortsätta att leva och dela sig – i stället för att dö av aktiverat p53. Eftersom Wig-1 stabiliserar p53 samtidigt som den stabiliserar faktorer som bidrar till tillväxt så kan Wig-1 också öka cellens beredskap om skadan inte lagats ordentligt.

Original publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

Paper I:

Prahl M., **Vilborg A.,** Palmberg C., Jörnvall H., Asker C., and Wiman K.G., The p53 target protein Wig-1 binds hnRNP A2/B1 and RNA Helicase A via RNA, *FEBS Lett,* 2008, 582, 2173–2177.

Paper II:

Vilborg A., Glahder J.A., Wilhelm M.T., Bersani C., Corcoran M., Mahmoudi S., Rosenstierne M., Grandér D., Farnebo M., Norrild B., and Wiman K.G., The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element, *Proc Nat Acad Sci*, 2009, 106, 15756 –15761.

Paper III:

Vilborg A., Bersani C. and Wiman K.G., The p53 target Wig-1 regulates N-Myc mRNA levels and maintainins the de-differentiation phenotype of N-Myc amplified neuroblastoma cells, *Manuscript,* 2010.

Paper IV:

Vilborg A., Rozell B., Bersani C., Wiman K.G. and Wilhelm M.T., The p53 target gene Wig-1 is essential for early embryonic development, *Manuscript*, 2010.

Related publication

Vilborg A., Wilhelm M.T. and Wiman K.G., Regulation of tumor suppressor p53 at the RNA level, *J Mol Med*. 2010, 88(7), 645-52

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List of abbreviations

53BP1 – 53 binding protein 1
ALS – Amyotrophic lateral sclerosis
AMD – ARE mediated decay
ARE – Adenosine/Uracil (AU) rich elements
ARE-BP – ARE binding protein
ARF – Alternate reading frame
ATM – Ataxia telangiectasia mutated
ASPP 1/2 – Apoptosis-stimulating protein of p53 1/2
ATR – Ataxia telangiectasia and Rad3 related
AUF1 – AU-rich element RNA-binding protein 1
Bax – Bcl-2-associated X protein
Bcl-2 – B-cell lymphoma 2
Bcl-6 – B-cell lymphoma 6
BH3 – Bcl-2 homology domain 3
Bmi-1 – B lymphoma Mo-MLV insertion region 1
Cdc2 – Cell division control protein 2 homolog
CDK – Cyclin dependent kinase
CED-1 – Cell Death abnormality-1
DDR – DNA damage response
DDX5 – DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
DGCR8 – DiGeorge syndrome critical region gene 8
DNA – Deoxyribonucleic acid
dsDNA – Double stranded DNA
dsRNA – Double stranded RNA
ESC – Embryonic stem cells
Fbw7 – F-box and WD repeat domain containing 7
FGF-2 – Fibroblast growth factor 2
G1 – Gap 1
G2/M – Gap 2 /Mitosis
GADD45 – Growth Arrest and DNA Damage
GLD-1 – Defective in Germ Line Development 1
HectH9/Huwe1 – HECT, UBA and WWE domain containing 1
hnRNPA2/B1 – Heterogeneous nuclear ribonucleoprotein
HOXA5 – Homeobox protein Hox-A5
HuR – Human antigen R
HZF – Haematopoietic zinc finger
iASPP – Inhibitor of ASPP
iPSC – Induced pluripotent stem cell
IRES – Internal ribosomal entry site
IVF – *In vitro* fertilization
JAZ – Just another zinc finger protein
kDa – Kilo Dalton
LIF – Leukemia inhibitory factor
Mdm2 – Mouse double minute 2
miRNA - MicroRNA
mRNA – Messenger RNA
MLE - Maleless
ORF – Open reading frame
PABP – PolyA binding protein

PAG608 – p53 activated gene 608
piRNA – Piwi-interacting RNA
PKCδ – Protein kinase C, delta
polyA – Poly adenosine
pRb – Retinoblastoma protein
PUMA – p53 upregulated modulator of apoptosis
RHA – RNA Helicase A
RISC – RNA-induced silencing complex
RNA – Ribonucleic acid
RPL26 – 60S ribosomal protein L26
S – Synthesis
siRNA – Small interfering RNA
Skp2 – S-phase kinase-associated protein 2
ssDNA – Single stranded DNA
TRRAP – Transactivation/transformation associated protein
TTP – Tristetraproline
UTR – Untranslated region
Wrap53 – WD40-encoding RNA antisense to p53
Wig-1 – Wild type p53 induced gene 1
ZMAT3 – Zinc finger matrin type 3

Introduction

What is cancer?

This question is obviously a handful to answer. In the short version, cancer is either uncontrolled and untimely cell growth, or prolonged survival of cells that should be dying, or both. A cell should know when, how much, and how many times to divide, it should respect borders, and it should die if so instructed. Normal cells know how to behave from integrating a myriad of signals and factors, some inherent to the particular cell type and some based on external circumstances – contacts with neighboring cells and the local environment but also signals from far away, mediated through the blood stream. In order to turn into a cancer cell, the cell has to render itself insensitive to these signals and factors. They achieve the escape from control through acquiring a number of traits, elegantly summarized by Hanahan and Weinberg in their seminal paper "The Hallmarks of Cancer"[1]. The cell can overcome the barriers represented by these hallmarks in various ways. They can activate genes that promote growth, invasion, and angiogenesis (genes that we know as *oncogenes*), or inactivate genes that prevent these events (genes known to us as *tumor suppressor genes*). This (in-)activation can occur by genetic changes such as mutation of a gene, gene amplification or deletion, or by modifying chromatin (epigenetic changes). Sometimes, the inactivation of one single gene can contribute to overcoming several of Hanahan and Weinberg's hallmarks. Such is the case for p53.[*]

p53

One of the main characters of this thesis will be p53, arguably the most studied gene in history. Its most important biological role is to prevent transformation of normal cells into cancer cells. Simple as that may sound, the function of p53 is extremely complex. p53 serves as the hub in a vast network – or rather in several different networks – receiving a flood of input on potential threats to the cell and responding in ways designed to keep the cell from turning cancerous. p53 has been bestowed the name "the guardian of the genome"[2], a name that reflects the important job p53 does protecting us from cancer. This protective role is demonstrated by the fact that virtually all tumors have inactivated the p53 pathway in one way or another – often by p53 mutation; something between 30-50% of human tumors carry mutated p53 (www-p53.iarc.fr; p53.free.fr). Additionally, p53 mutations are implied in inherited cancer since the highly tumor prone Li-Fraumeni syndrome is caused by germ line p53 mutations[3] (reviewed in[4]). The tumor suppressive role of p53 is also shared with our murine cousins – mice lacking p53 rapidly develop tumors and die before nine months of age, typically from lymphoma and sarcoma[5].

[*] At this point I will introduce my footnotes, which I have included for those brave few who might attempt reading this thesis without being familiar with scientific jargon. As an introduction: this thesis deals a lot with *transcription factors* and *mRNA regulation.* In the nucleus, a gene is *transcribed* into RNA – a process regulated by *transcription factors.* This RNA can be an mRNA, which is then exported to the cytoplasm where it is *translated* by the ribosome into a protein, which carries out functions in the cell. The RNA can also be some other kind of RNA (like a microRNA) that, after some processing, carries out its function as an RNA molecule.

p53 was identified in 1979 by Lane and others[6-9] as a protein bound to the SV40 virus Large T antigen, and was first thought to be an oncogene – the gene subsequently cloned[10-12] was a mutated version of p53 that could function as a dominant negative to the wild type (reviewed in[13]). The misunderstanding was resolved when wild type p53 was found to be frequently deleted in colorectal cancer[14], suggesting that it was a tumor suppressor. This suggestion was confirmed by a number of studies in human and rodent cells *in vivo* and *in vitro* (reviewed in[13]).

Since the discovery more than 30 years ago the knowledge on p53 has virtually exploded[15]. Judging from the literature today, p53 is involved in any and every pathological and physiological process imaginable, from cancer to embryonic development and aging, passing by any number of diseases on the way. All I can manage here is to briefly brush the surface of p53 knowledge – starting out with one fundamentally important aspect of p53: It is a protein that is activated by stress.

p53 is activated by stress

In healthy unstressed cells, p53 levels are kept low, almost undetectable. After cellular stress p53 accumulates dramatically, largely due to stabilization of the p53 protein. This stabilization is mainly achieved by blocking the interaction between p53 and its negative regulator Mdm2 – a process described in detail below. p53 can be activated by a diverse array of signals including DNA damage, oncogene activation, nutrient deprivation, hypoxia, and ribosomal stress. Also the very low presence of p53 in unstressed cells can exert functions, and these functions may be radically different from those initiated by high stress-induced p53 levels (reviewed in[16-18]). Here, I will limit the discussion to the p53 activators DNA damage and oncogene activation.

DNA damage, caused by, for instance, radiation or cytostatic drugs, leads to the accumulation and activation of protein complexes at the site of damage. These protein complexes in turn activate the kinases[*] ATM or ATR. ATM is activated by double stranded (ds) DNA breaks and ATR is activated by stalled replication forks[#] caused by single stranded (ss) DNA breaks or other DNA damage. Once activated, ATM and ATR will phosphorylate and activate the kinases Chk2 and Chk1, respectively, in that way amplifying the signal. Chk2 or Chk1, together with ATM or ATR will also phosphorylate sites on p53, which prevents the interaction between p53 and Mdm2 – in that way preventing the negative regulation of p53 (see below) (reviewed in[19]).

Another evident threat to the cell is the *activation of oncogenes* – genes that induce and/or promote cell proliferation. Different oncogenes can affect proliferation through many

[*] A kinase is a protein that phosphorylates – i.e. puts phospho-groups on – other things.

[#] A stalled replication fork has nothing to do with lazy eating but is instead a term to explain that during DNA replication – copying of the DNA – the replication machinery stops, usually because it encounters some problem (a DNA break or some other DNA damage, for example) and doesn't know what to do about it.

different mechanisms, but in the end they all result in the activation of growth promoting transcription factors, mainly of the E2F family. The E2Fs will then activate many genes involved in cell proliferation – in short; they work as powerful gas pedals for the cell. As such they also need to come with an emergency brake – a function carried out by p14ARF. p14ARF is a transcriptional target of E2F[20] (and of some other growth promoting transcription factors as well, for good measure). The p14ARF protein binds to Mdm2, and this binding will prevent the latter from binding to p53[21,22] (reviewed in[23]). While Mdm2 is thus otherwise engaged, p53 is free to exert its functions.

In a combination of the two above-mentioned mechanisms, activated oncogenes also induce a process known as the *DNA damage response* (DDR). Activated oncogenes drive deregulated replication, which causes stalled replication forks and double stranded DNA breaks – in turn inducing the ATR/ATM pathway of p53 activation[24,25].

See figure 1 for an illustration.

p53 function

How can any one protein have such profound impact on the cell as p53 apparently has? The main and most well studied function of p53 is that of a *transcriptional activator*– it activates the transcription of target genes, which will result in the production of other proteins and microRNAs. These p53 targets will in turn execute the p53 functions – and activation of a different subset of targets can alter the nature of the p53 response. The importance of transactivation for the ability of p53 to prevent transformation is underlined by the high mutation frequency in the p53 DNA binding domain, which is necessary for the transactivating activity (reviewed in[26]). When functioning as a transcriptional activator, a p53 tetramer binds DNA through its DNA binding domain. It binds to the p53 consensus response element, which is made up of two tandem copies of the motif "RRRCWWGYYY" separated by a spacer of 0 to 13 base pairs ("R" represents purines[*], "W" represents adenine or thymine, and "Y" represents pyrimidines[#]) (reviewed in[27,28]). p53 recruits the general transcription machinery to the promoter-enhancer region of its target genes, thus inducing their transcription[29,30] (reviewed in [31]).

Apart from being a transcriptional activator, p53 can also work as a *transrepressor* inhibiting the transcription of target genes – a classical example is that of the anti-apoptotic bcl-2 gene[32]. There appears to be multiple ways through which p53 can repress transcription, some of which are mediated through direct binding of p53 to DNA (reviewed in[33]).

Further, p53 can act *independently of transcription*. It can relocalize to mitochondria and directly affect the apoptotic process there[34] (see below for more on apoptosis) (reviewed in[35]).

[*] Purines: Adenine and Guanine

[#] Pyrmidines: Thymine and Cytosine

Further, p53 seems to be directly involved in RNA binding and microRNA processing[36] (see below for details).

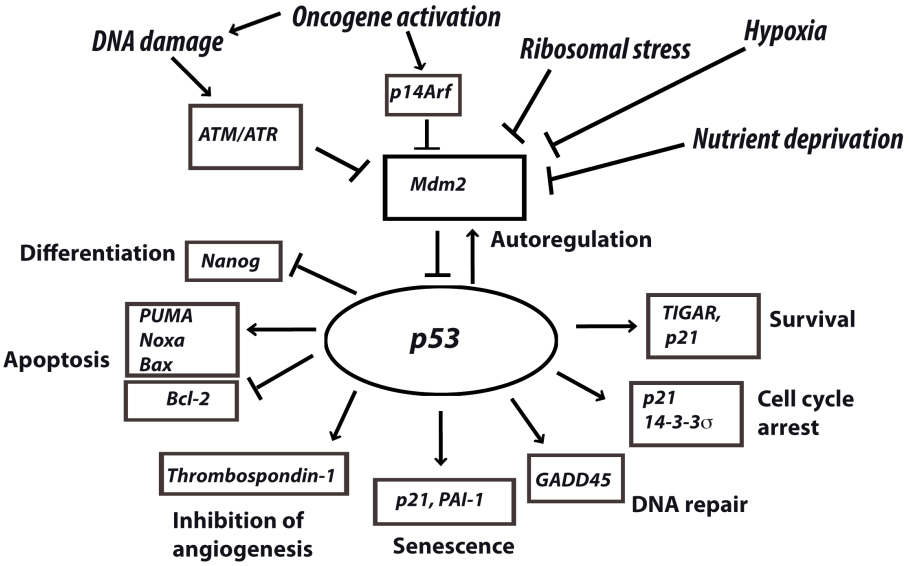

Figure 1: Overview of inputs to p53 and p53 responses, with examples of target genes. See text for details.

Outcomes of p53 activation

p53 induces cell cycle arrest and apoptosis

Once p53 has been activated and in turn has induced the transcription of a set of target genes – then what happens? The list of possible outcomes of p53 activation is constantly growing, but let's start with the classics – apoptosis and cell cycle arrest. Apoptosis is the process of programmed cell death – cellular suicide on command. Cell cycle arrest is somewhat less dramatic – the cell stops in its division program, to check and repair any potential damage. There are fewer p53 target genes involved in cell cycle arrest than apoptosis, the two main players here being p21[37] and 14-3-3σ[38] (reviewed in[39]). The p21 protein halts the cell cycle primarily through binding to and inhibiting the actions of cyclin dependent kinase (CDK) 2. CDK2 functions in a complex with cyclin E to enable the cell to pass the restriction point of the cell cycle, allowing the cell to proceed from the gap 1 (G1) phase to the synthesis (S) phase and start replicating its DNA. CDK2/Cyclin E achieves the passing of the restriction point by phosphorylating pRb, a protein that once phosphorylated will release the E2F transcription factors. The E2Fs then activate target genes involved in – among other things – DNA replication. Once the cell has passed the restriction point it is committed to division and will, in the absence of any major catastrophes, complete a round of DNA replication and cell division (reviewed in[40]). 14-3-3σ instead inhibit the activity of Cdc2 (also known as CDK1) and by this prevents the G2/M transition (reviewed in[41]).

p53 can also induce the expression of a wide variety of genes involved in apoptosis. One example is the death receptor Fas[42], which is important for the extrinsic pathway of apoptosis. This way of inducing apoptosis starts with activation of death receptors on the cell surface, which in turn activate caspase 8 that activates caspase 3, an effector caspase. Effector caspases then dismantle various cellular structures, destroying the cell in an orderly fashion. Among other pro-apoptotic p53 targets we find the BH3 proteins Bax[43], PUMA[44] and NOXA[45], mainly involved in the intrinsic apoptotic pathway. This apoptotic pathway starts with the permeabilization of the outer mitochondrial membrane, releasing cytochrome c and other constituents of the so-called apoptosome, which once formed activates effector caspases such as caspase 3 and 6. Bax is involved in the permeabilization of the outer mitochondrial membrane, and PUMA and NOXA prevents the Bax inhibitor Bcl-2 from binding to Bax (reviewed in[26,46]).

Evidence points to the p53 pro-apoptotic function as being the most important for tumor prevention. p21 knockout* mice, which have a severely impaired G1 arrest checkpoint, are not particularly tumor prone[47], indicating that p53 dependent cell cycle is not required for tumor prevention. However, knock-in# mice carrying a p53 mutant that is unable to activate apoptosis but retains some ability to activate cell cycle arrest is less tumor prone than p53 null mice[48], indicating that in the absence of apoptosis, cell cycle arrest can still contribute to tumor prevention.

Among other p53 responses we find *senescense* – irreversible cell cycle arrest. The cell remains viable and metabolically active but will not divide even in the presence of all the appropriate signals. There are two types of senescence: Replicative senescence and premature senescence, and p53 is involved in both types. Replicative senescence is activated by telomeric signals telling the cell that its chromosome ends (telomeres), which are shortened every time the DNA is replicated, are now too short to sustain further division. The shortened telomeres activate the ATM and ATR DNA damage response, which in turn activates p53. Premature senescence is induced in response to signals including DNA damage, oncogene activation, or reactive oxygen species. There are several p53 targets involved in senescence, one of which is p21 (reviewed in[49-51]), and senescence induction is one way for p53 to prevent clonal expansion of insipient tumor cells[52].

p53 can also regulate *differentiation* – the process when a stem cell or progenitor cell turns into a more specialized cell type. Stem cells are often resistant to cell cycle arrest and apoptosis, making them risk factors for tumorigenesis[42]. p53 may promote differentiation of stem cells into cell types that *can* undergo cell cycle arrest and apoptosis, as well as prevent self-renewal of stem cells[53] (reviewed in[50,54]). Further, p53 has been shown to be important for maintaining genomic stability in stem cells[55]. Efforts are being made to convert somatic cells§ to stem cells – so-called inducible pluripotent stem cells, iPSCs (a process of great therapeutic interest, for example to generate compatible donor tissue), and the factors required

* A knockout mouse is a mouse lacking one gene, in this case p21.

A "knock-in" mouse refers to a mouse were a gene has been replaced by another; in this case the wild-type p53 gene has been replaced by a mutant form of p53.

§ Somatic cells: Every cell in the body except for germ cells and undifferentiated stem cells.

for this transition are often oncogenes or suspected oncogenes. As such they will activate p53 through p14Arf. For that reason, p53 nullity contributes to the reprogramming of a somatic cell into a stem cell[56] (reviewed in[54]). The role of p53 in *embryonic development* has also been studied. p53 seems dispensable for normal embryonic development since most mice that lack p53 develop without problems[5], with the exception of a fraction of females that develop exencephaly[*57,58]. Additionally, p53 null mice have fewer offspring since they have lower levels of the p53 target LIF, important for implantation of the embryo into the uterus wall[59].

p53 can promote several forms of *DNA repair*, both through the transactivation of target genes (one example being GADD45 that is thought to remodel chromatin in order to make the DNA damage sites more accessible[60]) and through other mechanisms. p53 mainly promotes nucleotide- and base excision repair – where mismatches are identified, removed, and corrected – and mismatch repair, which repairs mistakes made during DNA replication. p53 also regulates the dsDNA break repair mechanisms; homologous recombination and non-homologous end joining. This regulation is exerted mainly transcription independent and is mainly negative, since these mechanism may increase genomic instability. In the case of severe damage such as dsDNA breaks, p53 is more likely to induce apoptosis. (Reviewed in[50,61]).

Inhibition of angiogenesis – the process through which tumors ensure sufficient blood supply to bring them oxygen and nutrients is inhibited by p53 through the activation of anti-angiogenic thrombospondin 1[62].

Paradoxically, p53 can also induce genes involved in promoting *survival.* One of these genes is p21 – while being pro cell cycle arrest, p21 antagonizes apoptosis. The list also includes several antioxidant genes and genes involved in metabolism. The logic behind this surprising p53 function may be that the elimination of every cell ever exposed to any kind of stress is not desirable – while protecting us from getting cancer; it would lead to tissue degeneration (reviewed in[16,63]).

p53 has been implicated in several *other diseases* apart from cancer, generally diseases connected to excessive cell death. These include diabetes, cell death after ischemia, and various neurodegenerative diseases such as Huntington, Parkinson, and Alzheimer (reviewed in[16,17]). Since p53 is able to eliminate cells through apoptosis and senescence and to induce differentiation, thus reducing stem cell populations, one is tempted to assume a role for p53 in *aging* as well. Initial studies in mice expressing constitutively active p53 also seemed to confirm this hypothesis[64]. However, subsequent mice models carrying an extra copy of p53 but under control of its normal regulatory elements demonstrated that properly controlled p53 did not induce aging. Instead it actually promoted longevity, largely by preventing tumorigenesis[65]. (Reviewed in[66]).

See figure 1 for illustration.

[*] Exencephaly: the brain is localized outside the skull

How does p53 choose?

Since p53 has a huge number of target genes and an equally vast repertoire of possible responses at its service, how can it possible know what to choose? The simplest explanation is that the p53 *affinity to its response elements* decides – some genes harbor perfect p53 consensus response elements located within favorable regions relative to the transcription start site, while other genes carry less well placed response elements with mismatches. In general, cell cycle arrest genes tend to have high-affinity response elements and pro-apoptotic genes have low-affinity response elements. Further, *p53 abundance* (more p53 favors a pro-apoptotic response) *and post-translational modifications* of the p53 protein impact promoter selectivity. There are also several p53 *cofactors* that direct p53 to certain promoters. For example, the ASPP1 and 2 proteins direct p53 activity towards pro-apoptotic target genes (there is also an inhibitory family member, iASPP). Further, 53BP1 is involved in directing p53 to cell cycle arrest and DNA repair genes, and HZF biases p53 to activate cell cycle arrest associated genes. Taken together, these p53 rules-of-choosing suggest that low stress levels lead to a p53 response centering on arrest/repair/recovery while high stress induces apoptosis or senescence. (Reviewed in[16,17,31,67]). Basal p53 levels, in the absence of any stress, seem to be enough for induction of physiological responses, such as fertility, development, metabolism, and stem cell maintenance. On the other hand, p53 activation and the resulting increase in p53 levels are necessary for its tumor preventive effects. (Reviewed in[15,67]). See figure 2 for illustration.

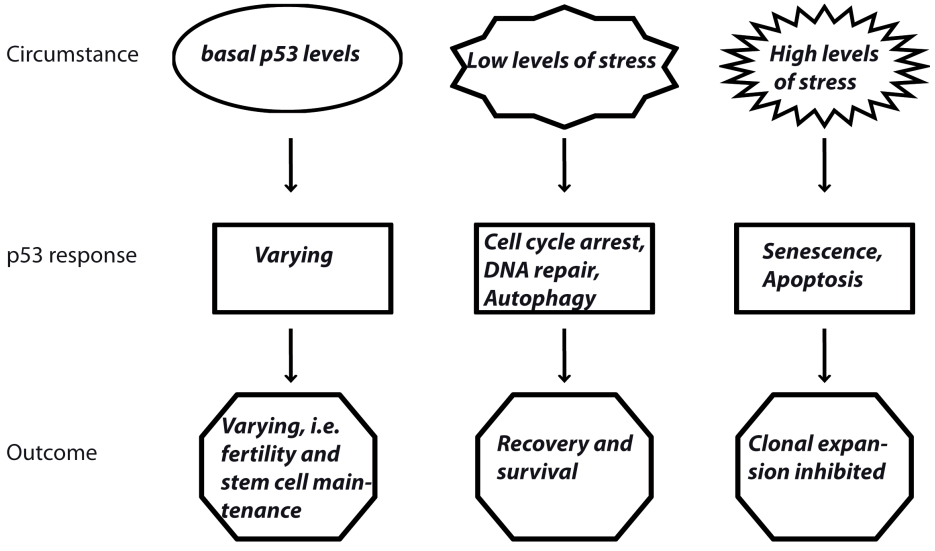

Figure 2: The various p53 responses to different amounts of stress, and the respective outcomes of those responses.

p53 family members and isoforms

To make matters a bit more complicated, there isn't just one of p53. p53 itself exists in several isoforms*. Some of these lack the N-terminal transactivation domain and can act as negative regulators of p53 function. Additionally, alternative splicing can give rise to different C-terminal variants. The exact function and physiological significance of these isoforms remain fairly unclear (reviewed in[51]). Apart from p53 itself, p53 also have two additional family members – p63 and p73. Although not as clearly connected to cancer as p53, there are several reports on the involvement of both p63 and especially p73 in cancer (reviewed in[68,69]). However, much interest has been directed to the obvious importance of these genes in normal development. p63 knockout mice die within hours after birth due to dehydration caused by lack of skin. These mice also have deficiencies in other epithelial tissues, and lack limbs[70,71]. p73 null mice are viable at birth, although a substantial amount of the pups die within the first three weeks of life. The homozygous knockouts have defect immune systems, have problems with chronic inflammation, and demonstrate brain malformations[72]. Both p63 and p73 come in a myriad of isoforms. Apart from the full-length isoforms (TA), they both exist in N-terminally truncated variants (ΔN), lacking the transactivation domain. The ΔN isoforms can act as negative regulators of the TA isoforms. In addition, there are a number of C-terminal isoforms generated by alternative splicing. (Reviewed in[68]).

Regulation of p53

Since p53 is licensed to make life-and-death decisions, it goes without saying that this protein is tightly regulated. Immediately after stress, when p53 levels need to rise quickly, regulation of the protein is predominant. Transcriptional and post-transcriptional regulations are likely to be important for maintaining steady-state levels of p53 and for prolonged stress responses.

Regulation of p53 at the protein level

p53 is a labile protein that is continuously synthesized and degraded. Thus, p53 levels can quickly be increased simply by halting degradation – a process overseen by Mdm2[73-75]. This negative regulator of p53 is an E3 ubiquitin ligase, binding to p53 and attaching the small protein ubiquitin to it[76]. Poly-ubiquitination marks p53 as a substrate for the proteasome, the cell's protein degradation machine. Further, Mdm2 is itself a transcriptional target of p53[77] – thus these two players are locked in a negative feedback loop where p53 activates the transcription of Mdm2 and Mdm2, once produced, induces the degradation of p53. Apart from being involved in p53 degradation, the Mdm2 binding to p53 also masks the p53 transactivation domain, thus preventing p53 from activating target genes. (Reviewed in[78,79]). The importance of the negative regulation of Mdm2 on p53 is underlined by the fact that Mdm2 knockout mice die during embryonic development due to excessive apoptosis, a phenotype that is completely reversed if backcrossed to p53 null mice[80,81]. Additionally, as

* Isoforms: different forms of one protein, generated from the same gene but processed into slightly different proteins that may or may not have different functions.

already mentioned, many of the ways to activate p53 after stress rely on interfering with the binding of Mdm2 to p53. Therefore it is perhaps not surprising that many tumors with wild-type p53 instead have increased levels of Mdm2 – for example, Mdm2 amplifications are found in about one third of sarcomas[82].

Mdm2 also has family – MdmX (also known as Mdm4), an Mdm2 homologue that binds to p53 and prevents it from transactivating target genes. However, MdmX does not target p53 for degradation and is not itself a p53 target. The interplay between Mdm2, MdmX and p53 is being studied intensely, but is as yet not completely elucidated. (Reviewed in[79,83]).

Apart from Mdm2 and MdmX, there are many other proteins shown to bind and regulate p53 in any variety of ways. On top of that, there are a vast number of different post-translational modifications such as phosphorylations, acetylations, ubiquitinations , methylations and so on that can affect p53 function, location, stability, choice of partners, and activity. The importance of these are yet debated, since knock-in mice mutated for individual phosphorylation sites show now evident phenotype (reviewed in[79]).

Transcriptional regulation of p53

The transcription of the p53 gene itself is in turn subject to regulation. The transcription factors PKCδ and HOXA5 have been implicated to transactivate[84,85], and the proto-oncogene* Bcl-6[86] to transrepress, p53. Moreover, *in vivo* evidence suggests that p53 can regulate its own expression, since loss of one p53 allele in mice results in a fourfold reduction of p53 mRNA and protein levels, as compared to p53 wild type mice[87].

Somewhat less is known about regulation of the p53 mRNA levels. This form of regulation – of p53 and of other genes – is the main focus of my thesis. Before discussing what is known about regulation of the p53 mRNA, I will spend some time describing mRNA regulation in general.

Regulation mediated through mRNA

An mRNA consists of the 5'untranslated region (UTR), the open reading frame (ORF) that codes for the protein, and the 3'UTR. The 5'UTR terminates in an atypically attached guanine mono phosphate to generate a structure known as the 5'cap, which protects the 5'UTR from degradation (reviewed in[88]). Additionally, the 5'cap and the proteins bound to it are recognized by the ribosome to initiate translation. The 5'UTR can also contain regulatory sequences, such as internal ribosomal entry sites (IRESes), which can provide alternative translation initiation. (Reviewed in[89]). The 3'UTR is protected from degradation by what is known as the polyA tail at its end. The polyA tail consists of approximately 250 adenosines[90], which are not encoded in the corresponding gene, but are added by a 3'end processing machinery that recognizes polyadenylation signals in the nascent mRNA. The polyA tail is

* Proto-oncogene: a potential oncogene carrying out its normal function in the cell.

bound by polyA-binding proteins (PABPs) that assist polyadenylation, export, translation and stability of the mRNA (reviewed in[91,92]).

The steady state level of any given mRNA is the net difference of what is being produced and what is being degraded. In order for changes in transcription rates to quickly affect protein levels, the mRNA coding for that protein needs to be both transcribed and degraded at a high rate (that is, have a short half-life). Logically, mRNAs coding for proteins with important regulatory functions generally have the shortest half-lives, reflecting a need to quickly change the abundance of these proteins. mRNA degradation is thus an important process for controlling central regulatory functions in the cell. The removal of the protective 5'cap and the polyA tail are rate limiting for degradation to occur. These steps are required for the exonucleases* responsible for degradation to gain access to the mRNA ends. In mammalian cells, the main pathway for initiation of mRNA degradation seems to be through deadenylation. mRNA continuously loose their polyA tails, and once the tail has reached below a critical level (around 30-60 As), mRNA degradation commences. This process generally starts with hydrolysis of the 5'cap followed by 5'-3' degradation mediated by the exonuclease Xrn1. In an alternative pathway the mRNA, after loss of the polyA tail, is degraded from the 3'end by the exosome. mRNA degradation mainly takes place in the cytoplasm, although the exosome can also be found in the nucleus. (Reviewed in[93]). In order to make mRNA degradation a regulated process, deadenylation cannot happen at the same rate for all mRNA – and indeed it does not! Most elements regulating deadenylation – and other aspects of mRNA degradation and translation efficiency – reside in the 3'UTR of the mRNA in question. The most well known factors to regulate mRNA stability and translation are microRNA (miRNA) and AU-rich elements (AREs). See figure 3 for an illustration.

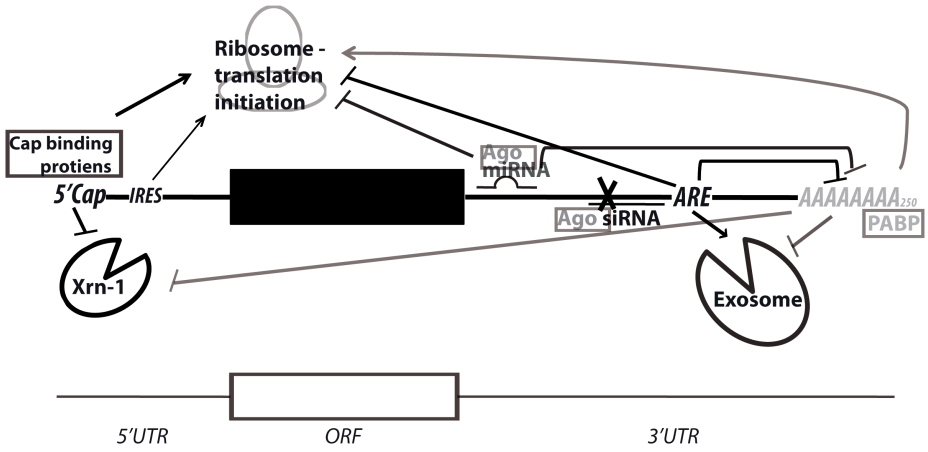

Figure 3: Factors regulating mRNA stability and translation efficiency

* Exonucleases: nucleases that degrades from the ends, as opposed to endonucleases, that cut in internal part of an RNA.

AU-rich elements and the proteins that bind to them – and how they affect p53

AU-rich elements are present in the 3'UTRs of certain mRNAs and consist of the element AUUUA in various constellations or regions of Us only[94,95]. AREs are found in five to eight % of the transcriptome*, generally in mRNAs of genes that need very precise control of their expression. Examples include genes encoding proteins regulating cell growth or response to external factors, such as c-Myc, N-Myc, cyclins, interferons, p53 and p21 (reviewed in[94,95]). AREs can mediate degradation both by increasing deadenylation and subsequent decay of the mRNA they reside in. Several exosomal subunits (for example PM-Scl-75), as well as the 5'-3' exonuclease Xrn1, can recognize and bind AREs. Both Xrn1 and PM-Scl-75 seem to be required for effective ARE-mediated decay (AMD). (Reviewed in[93,96]). Further, AREs can decrease the translation efficiency of the mRNA they reside in. Most factors reported to bind and regulate AREs affect the target mRNA negatively, by promoting degradation or inhibiting translation (a classical example being Tristetraproline (TTP)). There are also positive ARE regulators such as Human antigen R (HuR), and those that can act either positively or negatively, depending on the mRNA in question – for example AUF1[94,96]. ARE-interacting proteins are important nodes of regulation, since they can potentially control the levels of many different mRNAs in the cell. (Reviewed in[95,97]). The 3'UTR of p53 harbors one U-rich region (18 continuous Us), and one additional ARE containing the AUUUA motif[98,99], both of which are targeted for regulation by several ARE-binding proteins (ARE-BPs). The first reported factor affecting the p53 mRNA is a still unidentified 40 kDa protein that inhibits translation of p53 mRNA via the U-rich region[100]. In addition, the C. elegance protein GLD-1, related to the Quaking protein in mammals, binds to the 3'UTR of the C. elegance p53 homologue CED-1 and represses its translation[101]. Further, HuR binds to the p53 AREs and increases p53 mRNA stability[102] and translation[98]. However, the effect of HuR on p53 is complex, since a recent study of HuR knockout mice shows that HuR also can stabilize Mdm2 mRNA through an ARE, thus leading to decreased p53 protein levels[103]. See figure 4 for an illustration of factors regulating the AREs in p53 mRNA. p53, being a tumor suppressor, may appear as a surprising target for ARE-mediated regulation. Most AREs are found in genes associated with increased proliferation, suggesting that positive ARE regulation would promote tumor growth, and vice versa – that negative ARE regulation would cause tumor suppression (reviewed in[95,97]). Consistent with this notion, several studies have shown that HuR is overexpressed and/or relocalized to the cytoplasm (where it is active in ARE regulation) in cancer[104-107]. Further, the ARE destabilizer TTP has been shown to act as a tumor suppressor[108], and reduced TTP levels is a bad prognostic marker in breast cancer[109]. However, HuR can clearly also stabilize mRNAs involved in growth inhibition, such as p21[110]. Thus, ARE-mediated regulation is complex and may affect growth-inhibitory genes as well as growth-promoting ones. It is possible that other and yet to be identified regulatory pathways can enable selective regulation of ARE-containing mRNAs.

* Transcriptome: Everything transcribed (all the RNA) in a given cell. Often used to mean mRNA specifically.

miRNAs and how they regulate p53

microRNAs (miRNAs) and small interfering RNAs (siRNAs) are short RNAs (around 22 nucleotides) that were recently disovered[111] but are already widely recognized as important mRNA regulators. miRNAs are endogenous* RNAs transcribed as long miRNA precursors, while siRNA are either exogenous or produced endogenously by alternative pathways (known as endosiRNAs). miRNA precursos (termed pri-miRNA) are cleaved in the nucleus by the ribonuclease Drosha in a complex with DGCR8 and DDX5. The resulting, approximately 70 nt long, pre-miRNA has a hairpin structure with a two nucleotide 3'end overhang, and is exported to the cytoplasm by Exportin-5. Once in the cytoplasm, it is recognized and cleaved by Dicer to yield a mature, double stranded miRNA. One of the strands of the mature miRNA is incorporated in the RISC complex containing an Ago protein. The miRNA then guides the RISC complex to target mRNAs by binding to partially complementary sequences in the 3'UTRs of these mRNAs. The binding of targets leads to Ago-dependent inhibition of translation or exonucleolytic mRNA degradation. siRNA are recognized by Dicer and similarly incorporated into RISC complexes, but unlike most miRNA they are perfectly complementary to the target mRNA and directs endonucleolytic cleavage and subsequent degradation of the target mRNA. The 3'UTR of one particular gene can have many different miRNA binding sites, and one miRNA typically has many different targets[112-114]. The p53 3'UTR is regulated by several miRNA, namely miR-125a[95], miR-125b[115,116], miR-504[117], the miR-30 family[118], and miR-1285[119]. Moreover, miR-125b was shown to target upstream regulators of p53, leading to decreased transcription of the p53 gene as well[115]. See figure 4 for an illustration of miRNA regulating p53 mRNA. Various miRNAs have been indicated both as tumor suppressors (such as the miR-34 family) and oncogenes (such as the miR-17-92 cluster) (reviewed in[114]). However, miRNA function may be selected against during transformation since global miRNA levels often are decreased in cancer[120,121].

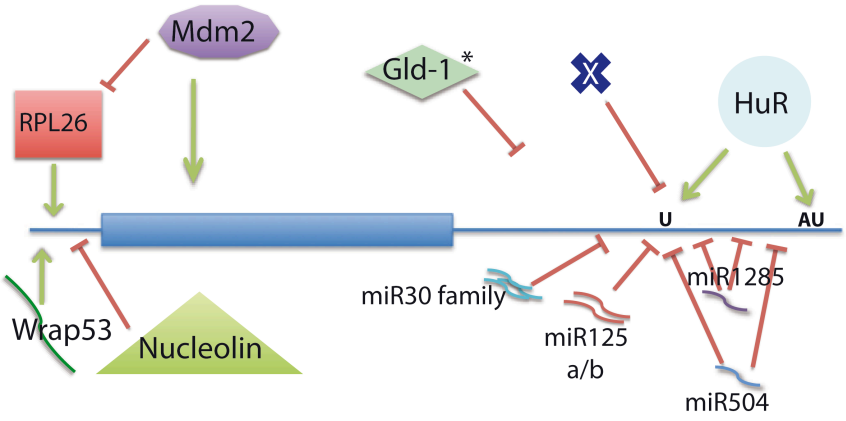

Figure 4: p53 mRNA and the factors that regulate it. Asterisk indicates a C. elegance protein. See text for details

* Endogenous – produced in the cell; as opposed to exogenous meaning that someone (like a scientist) put it there.

Interplays between miRNA and AREs

Nature seems to favor complexity whenever possible, so it is perhaps not surprising that ARE-mediated and miRNA-mediated effects on 3'UTRs are linked in many ways. A high AU content around the miRNA target site increases the likelihood of miRNA-mediated mRNA degradation, and miRNA target sites are often located in the vicinity of AREs.[122,123]. In addition, there are several examples of collaboration between AMD and microRNAs[124-126].

miRNA, AREs, and development

ARE-mediated regulation can play an important role during embryonic development, demonstrated by the embryonic lethal phenotype of the ARE-BP HuR, which is caused by deregulation of HuR targets[127]. AREs are also important in late stage oocytes and in the early embryo, where they regulate stability of the maternal mRNAs that need to be retained until the embryo genome is activated and transcription of embryo genes can commence (reviewed in[128]). The importance of miRNA during development is illustrated by the early embryonic lethal phenotype in Dicer[129] and DGCR8[130] knockout mice. Further evidence is found in the early post-natal lethality of mice knockout for the miR-17-92 cluster and the embryonic lethal phenotype of the simultaneous knockout of miR-17-92 and the paralogue* miR-106b-25 cluster[131]. Also interesting in terms of RNA in development are the piRNAs, germ cell specific small miRNA-like RNAs. The piRNAs associate with the Piwi subclass of Argonaut proteins and are essential for germ cell development. Their function is so far mainly unknown, but may involve silencing of transposons# (reviewed in[132]).

Post-transcriptional regulation of p53 through the 5'UTR and ORF

p53, being ever carefully regulated, is subject to control through its 5'UTR and ORF in addition to its 3'UTR. The p53 5'UTR is GC-rich and forms a stem-loop that hinders ribosome access during cap-dependent translation initiation. Cap-independent translation initiation using an IRES can overcome this difficulty[133]. The ribosomal protein RPL26 can enhance, and the protein Nucleolin can inhibit, p53 translation[134]. Further, the p53 5'UTR is stabilized by the antisense transcript Wrap53 (WD40-encoding RNA antisense to p53), a natural antisense RNA encoded by the Wrap53 gene located on chromosome 17, directly overlapping the first exon of p53[135]. The coding sequence of p53 mRNA also regulates p53 expression. The Mdm2 protein binds to nucleotides corresponding to amino acids 15-26 of p53 mRNA, a binding that both enhances p53 translation and inhibits the E3 ligase activity of Mdm2 (which in turn prevents p53 degradation), thus increasing p53 protein levels in several ways[136]. This is somewhat surprising considering the well-established role for Mdm2 as a negative p53 regulator, and emphasizes the complexity of the regulatory networks controlling p53 levels and activity.

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* Paralogue: a homologue (similar protein/RNA) within the same species.

Transposons are mobile elements in the DNA, able to move from one site in the genome to another – causing risk of mutations and alterations of the DNA in the process.

Role of p53 in posttranscriptional gene regulation

Interestingly, p53 itself can also regulate post-transcriptional gene regulation in several ways. p53 activates transcription of the miRNAs miR-34a, b and c that can trigger apoptosis (miR-34a), and reduce cell growth and/or induce cellular senescence (all of the miR-34)[137-141]. p53 also induces the transcription of the tumor suppressive miR-145[142]. In addition, p53 was recently shown to transcriptionally repress the miR-17-92 cluster after activation by hypoxia[143]. Further, loss of miRNA biogenesis in Dicer knockout mice activates p53 by upregulation of p19ARF (mouse homologue of p14ARF), resulting in p53 dependent senescence[144]. p53 can also enhance the biogenesis of several growth-inhibitory miRNAs post-transcriptionally by interacting with Drosha[36]. p53 may itself engage in regulation of 5'UTRs – according to one study, p53 can inhibit its own translation by binding to its 5'UTR[145]. Similarly, p53 has been shown to bind to the 5'UTRs of CDK4[146], and FGF-2 [147,148] and inhibit their IRES-dependent translation.

After this introduction to p53 and to mRNA regulation, I will move on to the p53 target gene involved in mRNA regulation that has been the focus of my studies – Wig-1.

Wig-1 – gene structure and expression

Wig-1 (for wild type p53-induced gene 1; also known as PAG608 and ZMAT3) is a direct transcriptional target of p53[149-151]. Human Wig-1 maps to 3q26.3-27, and the NCBI database contains two transcript variants of the Wig-1 gene. Isoform 1 (NM_022470) has a shorter 5'UTR due to lack of exon 2, and also has one extra amino acid in the C-terminal domain as compared to isoform 2 (NM_152240) due to alternative splice site usage. The Wig-1 ORF of 866 or 869 nucleotides (corresponding to proteins of 288 or 289 amino acids) is followed by a very long 3'UTR with three polyA sites (usage of the most distal gives an eight kb long 3'UTR). Apart form the full-length protein translated from the first ATG residing in a relatively weak context, there is a downstream in-frame ATG that can be used for translational initiation, giving rise to a protein lacking the 20 most N-terminal amino acids. The presence of an upstream ATG, usage of which translates into an out-of frame ORF that terminates downstream of the first in-frame ATG, suggests that the translation of this upORF would favor usage of the downstream in-frame ATG for translation of the Wig-1 protein[152]. The Wig-1 gene structure – two in-frame ATGs and an out-of-frame upORF – is conserved in mouse and chicken[152]. Human Wig-1 has a perfect consensus p53 response element in intron 1 (Wilhelm and Hellborg, unpublished results) and mouse Wig-1 contains two functional, but not absolute consensus, p53 response elements in the promoter region[153]. The Wig-1 protein contains a nuclear localization signal and three zinc fingers of the Cys_2His_2 type. The zinc fingers are unusual in their inter-histidine distance within the zinc fingers (five amino acids instead of the normal three to four) and their long linkers between the zinc fingers (56-75 amino acids compared to six to eight as in most other zinc finger proteins). See figure 5 for an illustration of Wig-1 gene and protein structure. Wig-1 is very highly conserved down to fish, especially with regard to the zinc fingers that are almost perfectly conserved. The unusual zinc finger structure is shared with a small group of double stranded RNA (dsRNA) binding proteins that lack consensus dsRNA-binding motifs. The most well-studied member of this

group is JAZ, a protein that binds to the dsRNA nuclear export receptor Exportin-5[154], and can positively regulate p53 transcriptional activity by binding to the p53 protein[155]. We have found Wig-1 expression in all cell lines tested to date (U2OS, MCF7, HeLa, ME-180, HCT 116 p53 +/+ or -/-, H1299, Saos2, SK-N-BE(2), Kelly, SHSY-5Y, SKNAS, HEK-293, IMR-90, human primary fibroblasts (HDF), and NIH-3T3) regardless of p53 status; however Wig-1 levels are enhanced after stress due to activation of p53 (unpublished results).

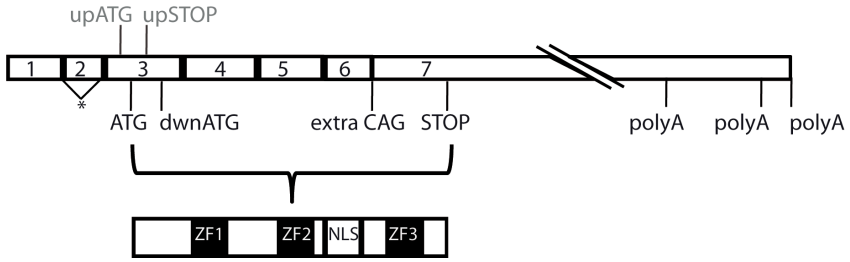

Figure 5: Human Wig-1 gene and protein structure. Asterisk indicates exon2, which is present in isoform 2 but not 1. dwnATG indicates the downstream in frame ATG. Extra CAG indicates the extra amino acid that is included in isoform 1 but not 2, due to alternative splice site usage. polyA indicates polyA site. ZF stands for zinc finger and NLS for nuclear localization signal.

Wig-1 is an RNA binding protein

In accordance with the function of its family member JAZ, Wig-1 is an RNA binding protein. Wig-1 binds both to longer dsRNA – a probe of around 130 bp was used in[156] – and to shorter RNA of 21 bp. In the latter case, Wig-1 only binds to short RNA that resemble miRNA, i.e. have a two-nucleotide 3' overhang. Both the first and second zinc finger of Wig-1 are necessary for binding to dsRNA in living cells[156,157].

Wig-1 and stem cells

Wig-1 may be involved in stem cell maintenance and support. One study, comparing genes enriched in both haematopoietic, neuronal and embryonic stem cells (compared to the corresponding differentiated cell types) found Wig-1 upregulated in all three stem cell compartments, indicating a role for Wig-1 in stem cells[158]. Another study showed upregulation of Wig-1 in haematopoietic stem cells null for Bmi-1, a factor necessary for the renewal of these cells[159].

Rat Wig-1: PAG608

The rat homologue of Wig-1 is constitutively expressed at relatively high levels in various regions of the rat nervous system, and has been shown to be induced in nervous system by a number of stress agents, including ischemia[160,161], treatment with methamphetamine[162], onset

of disease in a model of ALS[163], and by L-DOPA in a Parkinson model[164]. Some studies also suggest pro-apoptotic functions of PAG608 in stressed brain[160,165]. Taken together, these data indicate a role for PAG608/Wig-1 in neurological pathologies and stress responses.

For reasons that will later on become apparent, we will here take a quick detour to cover the RNA binding proteins RHA and hnRNPA2/B1

hnRNPA2/B1 and RHA

RNA Helicase A (RHA; also known as Nuclear DNA Helicase II) is a helicase unwinding both RNA and DNA. Its Drosophila homologue, MLE (maleless), is essential for maintaining the X chromosome hypertranscriptional*. In mammals RHA is involved in transcriptional regulation, for example by bridging the transcriptional co-activators CBP/p300 to RNA polymerase II[166-168]. (Reviewed in[169]). In addition, RHA has been shown to participate in the translation of selected mRNAs by recognizing a 5'UTR control element[170].

Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) are highly abundant, RNA-binding proteins derived from the same gene and differing by only 12 amino acids, due to the presence of exon 2 in the B1 transcript[171]. They are truly multitasking and their functions include packaging of nascent RNA, telomere biogenesis/maintenance, transcription, alternative pre-mRNA splicing, nuclear import and export, cytoplasmic trafficking of mRNA and regulation of translation (reviewed in[172]). hnRNPA2 has also been shown to be an ARE-BP capable of binding to AREs in the 3'UTRs of glucose transporter 1[173] and β-actin[174], affecting their mRNA stability and turnover.

From the title of my thesis it should be obvious that p53 is not the only famous transcription factor in Wig-1's acquaintance. We will now move on to the world of Myc.

Myc

Myc, as p53, is a tumor-associated gene with long history. The founding member of the Myc family, c-Myc, was first identified some 30 years ago as the cellular counterpart of the avian myelocytomatosis virus oncogene[175]. The Myc family later expanded when N-Myc[176,177] and L-Myc[178] were discovered in the beginning of the 1980's. While c-Myc is widely expressed in adult tissues, both of its cousins are almost exclusively expressed during embryonic development. (Reviewed in[175,179-181]). Still, their function is widely overlapping; hence in this text I will refer to all the family members jointly as "Myc", unless otherwise specified.

* Hypertranscriptional means exactly what it sounds like – the transcription increases. This renders the transcriptional output from the male one X chromosome on pair with that of the two X chromosomes of the female. This is how it's done is in flies – in mammals instead, one of the female X chromosomes is transcriptional inactive, and here RHA does not seem to be play a role.

Myc is a transcription factor

Myc is a transcriptional activator of the basic helix loop helix leucine zipper type*, and binds DNA in a heterodimer with its obligate partner Max. Myc and Max recognizes the so-called E-box sequence, the consensus of which is CACGTG. Even though Myc is absolutely dependent on Max for its function as a transcriptional activator, Max is not so faithful, but can also heterodimerize with other proteins such as Mad. Mad-Max heterodimers function as transcriptional repressors of E-boxes, thus opposing Myc function. Myc activates transcription by interacting with TRRAP (transactivation/transformation associated protein). Through TRRAP, Myc can recruit histon acetylation transferases responsible for acetylating the histones that DNA is wrapped around when packed in chromatin. Apart from TRRAP, Myc can also interact with a number of co-activator complexes that will induce other histone modifications. These modifications lead to the loosening up of chromatin structure, thus rendering the promoter in question more accessible for transcription. There seem to be few, if any, genes that rely exclusively on Myc for their transcription; rather, Myc tweaks the transcriptional landscape to a more favorable state, increasing the possibility for transcription to occur. Apart from its function as a transcriptional activator, Myc can also act as a transcriptional repressor, however in this case it does not act through E-boxes. (Reviewed in[175,179-181]).

What does Myc do?

The more appropriate question would be "What does Myc *not* do?". The E-box sequence is found at every 4000 bp of the genome. Many of these E-boxes are non-functional, but Myc is still estimated to regulate 10-15% of all genes[182,183]. Myc has a vast and extremely diverse set of targets, including genes transcribed by all three RNA polymerases – protein coding genes as well as various functional RNA molecules. (Reviewed in[179,180]). Myc regulates the *cell cycle* by transactivating several cyclins and CDKs, as well as inhibiting cell cycle arrest genes such as p21 (reviewed in[181]). This regulation leads to shortened G1 phase and enabling of G1 to S transition[184,185]. Further, Myc regulates *cell growth* in many different ways. It activates targets that increase the supply of the various molecules required for the cell to grow. It induces transcription of ribosomal genes – both those of ribosomal proteins and ribosomal RNA[186], and can in that way boost the cell's protein production. It also transactivates many genes involved in metabolism, leading to increased energy production. (Reviewed in[181]). Apart from these and many other potentially oncogenic Myc activities (such as promoting angiogenesis and metastasis), Myc can also induce *apoptosis.* Surprising as this may seem, it makes sense that Myc, being such a powerful cellular gas pedal, comes with an emergency brake. Myc induces apoptosis by transactivating p14ARF, which activates p53 through binding to and inhibiting Mdm2[187]. This mechanism implies that in order for Myc to transform cells, the apoptosis pathway needs to be somehow perturbed, for example by p53 mutation or Mdm2 overexpression. (Reviewed in [175,179,181]). However, lower Myc levels are required for driving proliferation than are needed for inducing apoptosis – probably to enable

* This refers to the structural elements in Myc involved in DNA binding.

Myc's physiological activities without killing the cell – but this dose dependency may render the Myc emergency brake somewhat inefficient[188].

Myc deregulation in cancer

Myc is deregulated in a majority of cancers in various ways. Myc can be activated by *viral transduction* – which is how it was discovered in the first place – meaning that viruses either carry their own copy of Myc or that they integrate in the host genome in a way that activates endogenous Myc. Myc can also be activated by *chromosomal translocations*[*], found in Burkitt's lymphoma. These translocations put c-Myc (on chromosome 8) next to immunoglobulin heavy or light chains (on chromosome 14, 2 or 22) in B-cells, and c-Myc ends up under the control of the immunoglobulin regulatory elements. Since B-cells are specialized in making antibodies, they produce very high amount of immunoglobulins, and the simultaneous regulation of the c-Myc gene leads to very high c-Myc levels as well. A third mechanism for Myc activation is *gene amplifications* – especially frequent for N-Myc in neuroblastoma, but also seen in other cancers such as lung cancer, where any of the Myc's can be amplified. (Reviewed in[175,180]).

Regulation of Myc expression

Myc is an immediate early gene, meaning that its gene expression is induced very rapidly upon stimulation with growth inducing signals. Conversely, anti-proliferative stimuli lead to a rapid shutdown of Myc expression. Transcription alone cannot explain the full change in Myc levels after growth "on or off" signals[189]. Both c-Myc and N-Myc are regulated through AREs in their 3'UTRs[190-195]. Further, the Myc 3'UTR is targeted by miRNAs. Both c-Myc and N-Myc are targeted by miR-34[196,197], and c-Myc is also a target of miR-145[142] (interestingly, both these miRNAs are transcriptional targets of p53[137-142]) and of miR-24[198] and let-7[126]. Further, the well known ARE stabilizer HuR, which can bind to the Myc AREs, actually represses c-Myc expression in a mechanism dependent on the miRNA let-7, presumably by recruiting let-7 to a target site adjacent to the HuR binding site[126]. c-Myc mRNA is also regulated through a coding region determinant RNA element in the coding region[199]. c-Myc protein levels are subject to proteasomal degradation induced by the E3 ligases Skp2 and Fbw7, and N-Myc is likewise targeted by the E3 ligase HectH9/Huwe1 (reviewed in[180]).

Myc and stem cells, differentiation, and embryonic development

c-Myc knockout mice die at E10.5[#] demonstrating multi-organ failure and general delay in development[200]. N-Myc knockout mice die before E11.5 and are also subject to delayed

[*] A chromosomal translocation means that two chromosomes break and are glued together with each other – resulting in two new, "hybrid" chromosomes.

[#] The days of embryonic development in mice are denominated "E" (for embryonic day) followed by a number, always ending with .5. This is because in order to keep track of the embryonic days, mice are put up for mating (i.e. male and female are put together) in the evening, and – hopefully, if the mood is right – do their thing

growth, in combination with defects in nervous system, lung and heart[201-204]. L-Myc knockouts, however, are viable without any obvious phenotype[205]. In addition to the clear role in gross embryonic development thus demonstrated by c-Myc and N-Myc, these two Mycs are also implied in stem cell biology. N-Myc is important for the maintenance of neurological stem cells[206] and c-Myc is necessary for the development of T-cells beyond the point when N-Myc expression in these cells is diminished[207]. Further, adult haematopoietic stem cells double knockout for both c- and N-Myc are not viable[208]. Myc has also been implicated in the reprogramming of somatic cells to inducible pluripotent stem cells, iPSCs[209]. Some somatic cells can be changed into iPSCs with the combined introduction of a few factors, of which Myc is one. Taken together, these observations indicate a crucial role for Myc in stem cells, confirmed by the phenotype of embryonic stem cells (ESCs) double knockout for both c- and N-Myc. These ESCs demonstrate reduced proliferation and survival, and when injected into blastocysts in an attempt to create chimaeric embryos* they either failed to generate embryos altogether, or in some cases generated embryos with very serious malformations. Thus, Myc function is necessary already at the pre-blastocyst stage of embryonic development[210] (reviewed in [211]).

N-Myc and neuroblastoma

Neuroblastoma is the most frequently diagnosed cancer among infants, and is responsible for 15% of deaths in childhood cancer[212]. It arises in the neural crest# derived precursors or immature cells of the sympathetic nervous system§. It is generally located in the adrenal gland or in a paraspinal location in the abdomen or chest[213,214]. N-Myc amplification occurs in 20-30% of neuroblastoma and is the most consistent marker of poor prognosis and aggressive disease (see fig. 6). It relates to poor outcome even in tumors with otherwise favorable disease patterns (such as localized disease)[212,214,215]. Further, mice overexpressing N-Myc in neural crest derived cells develop neuroblastoma, demonstrating that N-Myc expression can drive neuroblastoma formation[216]. Neuroblastoma may spontaneously differentiate[213], and it has been demonstrated that N-Myc knockdown using siRNA in neuroblastoma cells harboring N-Myc amplifications can induce cancer cell differentiation and apoptosis[217].

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during the following night. The next day will then be half a day of embryonic development, E0.5. The next day again will be E1.5, and so on. From this we learn that scientists, contrary to common belief, prefer not to dissect small animals during the night (the embryos removed would then be E followed by a whole number) but instead, whenever possible, work daytime.

* To make a knockout or knock-in mouse, the desired manipulations are carried out in ESCs, which are then injected into a blastocyst followed by implantation of the blastocyst in a female. The resulting embryo will have tissues generated both from the blastocyst cells and from the injected ESCs – a chimera.

The neural crest is a transient embryonic tissue in the developing nervous system. It arises from ectoderm when the neural tube closes (ectoderm is the outermost cell layer in the gastrula stage of the early embryo, and the neural tube is the embryo precursor of the central nervous system).

§ The sympathetic nervous system is a part of the autonomous nervous system responsible for the stress ("fright-and-flight") response.

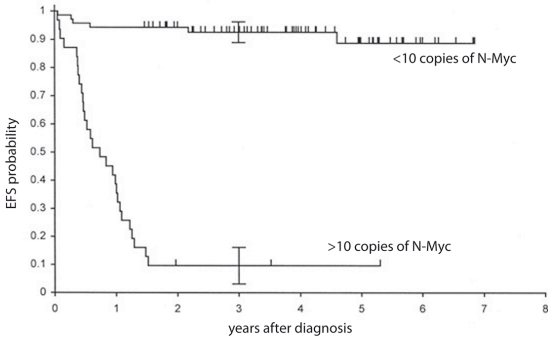

Figure 6: Probability of event free survival (EFS) in neuroblastoma patients with or without N-Myc amplification. From Scmidt et al., J.Clin.Oncol, 2000[215].

Aims of this thesis

The overall question asked in my thesis can be summarized in one very short sentence – "What does Wig-1 do?". Answering this question is important not only to learn more about Wig-1 itself, but also for gaining further insight into the function of the p53 tumor suppressor. Since p53 exerts its function mainly through transactivation of target genes, we must understand the function of these targets is order to fully understand the function of p53.

Specific aims of the papers:

Paper I

- To identify proteins interacting with Wig-1

Paper II

- To investigate the mechanism of, and function for, Wig-1 mediated regulation of p53

Paper III

- To study Wig-1 regulation of another ARE-containing mRNA – that of N-Myc – and the functional significance of this regulation

Paper IV

- To determine the role of Wig-1 in embryonic development

Results and Discussion

Paper I

At the outset of this paper little was known of the Wig-1 function. We knew that it was an RNA binding protein[156,157], however, earlier studies were based on assumptions made from the structural resemblance of Wig-1 to other RNA binding proteins and had been performed mainly *in vitro*. Albeit excellent approaches, these studies presented Wig-1 with RNA and analyzed binding or no binding, without addressing how important RNA binding is for the function of Wig-1 in living cells. Further, we knew that Wig-1, when overexpressed transiently, could somewhat reduce cell growth over longer periods of time[150]. To better be able to study the importance of Wig-1 RNA binding for Wig-1 function *in vivo*, we generated stable Saos-2-Tet-ON cells that overexpressed FLAG-tagged Wig-1, either wild-type or RNA-binding deficient zinc finger point mutants, after addition of doxycycline. We found that wild type but not mutant Wig-1 could inhibit cell growth modestly in long-term assays without any effect on cell cycle or cell death. Further, we used the FLAG-tagged Wig-1 to immunoprecipitate Wig-1 and identify co-purified proteins. In this way we found RHA and hnRNPA2/B1 to be bound to Wig-1 in an RNA dependent fashion.

Since both RHA and hnRNPA2/B1 are abundant RNA binding proteins with many roles in RNA function, biogenesis, and turnover in the cell (reviewed in[169,172]) it is hard to make any guesses of the function of Wig-1 based on its interactions with these proteins. However, knockdown of hnRNPA2/B1 using siRNA had been reported to cause G1-arrest and reduction in cell proliferation[218], and at this time we had started knocking down Wig-1 and noted a similar effect. To investigate this further, we knocked down either one alone or both together, and analyzed cell proliferation and cell cycle. We could conclude that knocking down both together had the same effect as knocking down either one alone – absence of an additive effect indicating that Wig-1 and hnRNPA2/B1 may function in the same pathway to affect cell cycle and cell viability.

In conclusion, from this study we learned that Wig-1 interacts with RHA and hnRNPA2/B1 through RNA. Whether this indicates a functional interaction, or if they just happen to sit on the same RNA frequently, remains to be shown. However, the fact that knock-down of either hnRNPA2/B1 or Wig-1 has the same effect on cell viability as knocking down both together suggests that these two proteins may share at least some functions.

Further, we found that both too much or too little Wig-1 has negative impact on cell viability, demonstrating the need for the cell to keep Wig-1 levels within appropriate range.

Even though this study failed to pin down an exact function of Wig-1, it demonstrated that RNA binding is important for Wig-1 *in vivo*. We performed an unbiased approach to investigate Wig-1 interactions, instead of assuming that these interactions were based on RNA. Still we came up with RNA binding as a key element, since the Wig-1 interaction partners identified bound Wig-1 through RNA. Also, we found that long-term overexpression of Wig-1 could decrease cell viability somewhat, but only if the Wig-1 used retained its RNA

binding ability. Clearly, the RNA binding capacity of Wig-1 is crucial for its function in living cells.

Paper II

This study started with the observation that Wig-1 knockdown using siRNA led to reduced p53 protein levels. We first confirmed that Wig-1 could positively regulate p53 protein levels both in unstressed cells and after p53 activation by DNA damage. We then investigated how this regulation affected the p53 stress response. Since both overexpression and knock-down of Wig-1 could decrease cell viability (Paper I) we performed these functional assays in a setting were we could analyze the effects of knocking down or overexpressing Wig-1 in p53 wild-type compared to p53 null isogenic cell lines. We took this approach to be able to separate the effect of Wig-1 on the p53 response to any other, p53 independent, effects of Wig-1. In this way, we could demonstrate that overexpression of Wig-1 potentiated the ability of p53 to inhibit colony formation after exposure to the DNA damaging agent camptothecin in HCT 116 cells, and that knockdown of Wig-1 reduced the p53 response to the DNA damaging agent mitomycin C in MCF7 cells.

Next, we set out to investigate the mechanism behind the Wig-1 regulation of p53. Since Wig-1 is an RNA binding protein[156,157] and RNA binding is important for its function (Paper I) we investigated if Wig-1 knockdown could affect the p53 mRNA. Indeed it could – p53 mRNA levels were decreased in the absence of Wig-1. We could further demonstrate that Wig-1 stabilizes the p53 mRNA by preventing its deadenylation, the first and rate limiting step in mRNA degradation (reviewed in[93]). Since mRNA degradation primarily takes place in the cytoplasm (reviewed in[93]), and exogenous Wig-1 was reported to be mainly nuclear[150,156], we investigated the localization of endogenous Wig-1 in detail. We found that Wig-1 is both nuclear and cytoplasmic, is able to shuttle between these two compartments, and is upregulated in both compartments after stress.

p53 mRNA contains two AREs: one U-rich stretch and one element containing the AUUUA pentamer[98,99]. p53 mRNA stability[102] and translation[98] are regulated through these regions, which prompted us to investigate if they were also implied in Wig-1 mediated regulation of p53 mRNA. We found that Wig-1 indeed binds to the U-rich ARE in p53 3'UTR, and regulates p53 mRNA stability through this region.

Our findings presented in this paper demonstrate that the p53 target Wig-1 acts in a positive feedback loop stabilizing p53 mRNA. We show that Wig-1 is important for maintaining the basal p53 mRNA levels, and also acts to augment the p53 response to cellular stress. This Wig-1-mediated stabilization of p53 mRNA may ascertain a sustained p53 response. Wig-1 can keep the p53 mRNA levels high enough to enable robust de novo protein synthesis, thus replacing the p53 degraded by Mdm2. In agreement with a role for Wig-1 in augmenting the p53 response, Wig-1 knockdown in rat neurons attenuates methamphetamine-induced p53-dependent cell death[162].

The fact that Wig-1 regulates p53 through binding to an ARE told us much about Wig-1 function – this finding placed Wig-1 among the ARE binding proteins that regulate mRNA stability and translation. Since most ARE binding proteins have multiple targets (reviewed in[94,97]) we hypothesized that this was true also for Wig-1. We performed a preliminary screen where we knocked down Wig-1 and investigated the levels of a number of proteins derived from ARE regulated mRNAs. In this screen we found c-Myc protein levels to be decreased upon Wig-1 knockdown – a finding that has kept us busy since.

Paper III

In paper II, we established that Wig-1 is an ARE-BP and found that knockdown of Wig-1 leads to decreased levels of c-Myc. Since both c-Myc and its family member N-Myc are regulated through AREs[192,193], we went on to investigate if N-Myc was regulated by Wig-1 as well. We knocked down Wig-1 in neuroblastoma cell lines carrying amplified N-Myc and found N-Myc protein levels to be decreased. This led to a more careful study of Wig-1 and N-Myc in neuroblastoma. We found that Wig-1 positively regulates N-Myc protein and mRNA levels and that Wig-1 binds to the 3'UTR of N-Myc and regulates it through an AU-rich region in the 3'UTR. N-Myc knockdown can lead to differentiation in SK-N-BE(2) cells[217], and thus we set out to investigate if Wig-1 knockdown could give a similar phenotype. And that was indeed the case – after Wig-1 knockdown for four days we could detect morphological changes associated with differentiation, G1-arrest, and upregulation of the differentiation marker NPY[219], comparable to what we observed upon N-Myc knockdown. This effect of Wig-1 on differentiation was dependent on N-Myc, since Wig-1 knockdown in another differentiation-competent neuroblastoma cell line lacking N-Myc expression (SHSY-5Y) did not induce differentiation.

This study raised the question why the p53 target gene Wig-1 would positively regulate an oncogene such as N-Myc. It is conceivable that Wig-1 acts independently of p53 during embryonic development, which is the major time for physiological N-Myc expression[212,214]. It is also plausible that Wig-1 target specificity may change after stress. Wig-1 targets a U-rich element in p53 mRNA and an AU-rich element in N-Myc mRNA, suggesting that the interaction of Wig-1 with the two AREs may be differently regulated. A third possibility is that Wig-1 belongs to the category of p53 target genes that promote survival (reviewed in[63]). I will get back to these questions in the final discussion.

Since loss of Wig-1-mediated regulation of N-Myc leads to differentiation of N-Myc amplified neuroblastoma cells there could be a therapeutic potential in interfering with this regulation. In tumors with mutant p53, decreasing Wig-1 levels or blocking Wig-1 RNA binding could be beneficial. In tumors with wild-type p53, it would be desirable to somehow direct Wig-1 away from the AU-rich element of N-Myc and instead increase its binding to the U-rich element of p53. Such a strategy would in one sweep remove the driving oncogene – N-Myc – and stabilize the killer – p53.

Paper IV

To gain further insight in the biological function of Wig-1, we set out to investigate the role of Wig-1 during embryonic development. First, we investigated Wig-1 expression in mouse embryos. We found Wig-1 to be expressed at high levels early during embryonic development (the earliest time point investigated by us was E5.5, but data from microarray studies indicate Wig-1 expression also before this point[220]). Levels then dropped somewhat to peak again at E10.5-13.5, after which point the expression decreased markedly. Immunohistochemistry revealed that Wig-1 is most highly expressed in brain and nervous system and in the fetal liver at E10.5-E13.5. Further, Wig-1 expression is independent of p53 during embryonic development, at least in unstressed embryos at E10.5 and E13.5, as revealed from immunohistochemistry of p53 knockout embryos.

To further study Wig-1 during embryonic development we generated Wig-1 knockout mice by targeted deletion of part of exon 2 and exon 3 and 4. The upstream ATG generating an upORF[152] was also mutated. We obtained germ line transmission form the injection of two separate ESC lines at a total of three times (one cell line was injected twice). The resulting chimaeric and heterozygous mice were phenotypically normal, although we acquired less than Mendelian ratio of heterozygous offspring from intercrossing heterozygous with wild type mice (around 37% instead of the expected 50%). Further, when intercrossing heterozygous mice with each other we could not detect any Wig-1 null offspring, indicating that Wig-1 knockout causes embryonic lethality. We obtained and genotyped embryos from different embryonic days but were unable to detect any knockout embryos at any point investigated. This directed out attention to the earliest phase of embryogenesis – the pre-implantation period from the zygote to the blastocyst stage of 32-64 cells. This phase normally takes 3.5 days in mouse. To determine if the knockout embryos were present at blastocyst stage, we performed *in vitro* fertilization (IVF) and grew zygotes in culture until they reached blastocyst stage, followed by genotyping of the blastocysts. Still, we found no knockout blastocysts, indicating that homozygous Wig-1 knockout in mice is lethal already before this time.

Mice knockout for the ARE-BP HuR were recently shown to die during embryonic development due to deregulation of several HuR targets[127]. Since we had shown Wig-1 to be an ARE-BP (Paper II and III), we asked whether the lethality observed in the Wig-1 knockouts could be explained in terms of deregulated Wig-1 targets. We knew that Wig-1 regulates N-Myc mRNA through and AU-rich region and that Wig-1 knockdown leads to decreased N-Myc mRNA and protein levels (Paper III). Further, we had shown that Wig-1 knockdown could decrease c-Myc protein levels (Paper II). Myc function seems to be required for the survival of ESCs and early embryonic development. c-Myc and N-Myc are redundant in ESCs and in the early embryo up until the point when Myc expression diverges[221]. A recent paper[210] demonstrated that the simultaneous knockout of both c-Myc and N-Myc in mouse ESC led to decreased survival and pluripotency and increased differentiation of these cells. When these ESCs were injected into blastocysts they either failed to generate any embryos or in some cases generated embryos with severe defects. This study is further supported by the finding that mice knockout for Max, the obligate partner for Myc, die at E6.5. Their lethality coincides with loss of maternal Max[222], indicating that Max/Myc function is required also before this stage. Therefore, we hypothesized that the

combined deregulation of c- and N-Myc could be the cause of the very early embryonic lethal phenotype of the Wig-1 knockout mice, and thus set out to confirm that Wig-1 does indeed regulate c-Myc. We showed that Wig-1 knockdown leads to decreased c-Myc mRNA and protein levels, and conversely, ectopically expressing Wig-1 increases c-Myc levels. Further, Wig-1 could bind to the 3'UTR of c-Myc, indicating that the regulation is mediated by an ARE in the c-Myc 3'UTR.

Interestingly, there is a mouse model where the c-Myc 3'UTR has been knocked out with retained c-Myc 5'UTR and coding region. This mouse is perfectly viable, arguing against an important role for regulation of the c-Myc 3'UTR during embryonic development[223]. However, AREs are generally considered to be destabilizing elements (reviewed in[97]), therefore, removing them may not result in a phenotype because c-Myc lacking its 3'UTR is stable and will be expressed at adequate levels for embryonic development to proceed. On the other hand, once the AREs *are* present, it may be necessary to keep them stabilized by the action of a protein such as Wig-1 in order to sustain c-Myc expression.

In addition to an effect in the pre-implantation embryo, loss of Wig-1 may affect germ cells, giving rise to low quality of Wig-1 negative oocytes and/or sperms. A role for Wig-1 in germ cells is in line with evidence from microarray studies (PubMed GeoProfiles GDS2112 and GDS1677), which show upregulation of Wig-1 in germ cells upon stimulation with growth factors. Evidence that Wig-1 is expressed from oocyte to blastocyst (with a peak in early blastocysts) can also be found in microarray data from Guo et al., *Develop. Cell*, 2010[220]. The same study reports expression of all the three Myc family members at these stages, and their expression pattern overlaps with that of Wig-1. The role of Myc in germ cells is not completely elucidated, but reports suggests that c-Myc is expressed during spermatogenesis[224] and that N-Myc plays an essential role for spermatogonial stem cell proliferation[225]. Further, c-Myc is expressed and may be involved in oocyte maturation[226,227], and at least *Xenopus*[*] c-Myc may be subjected to ARE-dependent degradation in oocytes, fertilized eggs, and early embryos[228,229]. Since Myc loss impairs survival already in the ESCs[210], it would not be surprising if decreased Myc levels could have a negative effect on germ cells. Alternatively, Wig-1 could regulate other ARE-containing mRNA in the germ cells, whose deregulation in the heterozygote Wig-1 cells could cause defects and/or death in these cells. ARE mediated regulation is important in late oocyte maturation and early embryonic development. At these stages, the oocyte/embryo genome is transcriptionally silent, and translation is sustained by maternal mRNAs. These mRNAs must be kept stable from the 2nd meiotic division, to which stage the oocyte develops during puberty and then arrests and stops transcription, until the embryo genome is activated (at the 2nd cell division in the mouse), which may happen months or even years later. Most maternal mRNAs are degraded in the late stage oocyte, but some that are crucial for the earliest embryonic development are retained until after embryo genome activation. AREs are important for regulating stability of these retained maternal mRNAs[128]. If Wig-1 is haploinsufficient, decreased Wig-1 levels in the oocyte can lead to deregulation of a number of these maternal mRNA required immediately before and after fertilization. In the case of defect Wig-1 heterozygote germ cells, the wild-type germ cells will take over, which

[*] *Xenopus* is a frog, good for research because of its huge, easy-to-manipulate eggs.

could explain our observation of skewed ratio of heterozygous versus wild type animals in normal litter sizes.

In conclusion, in this paper we show that Wig-1 knockout mice die during embryonic development before blastocyst stage, probably caused by deregulation of Wig-1 targets such as c-Myc and N-Myc.

What does Wig-1 do?

Combined discussion of all papers

The results presented in this thesis demonstrates that Wig-1 is and ARE-BP, a protein that binds to and regulates mRNA through AREs in the 3'UTRs of its target mRNAs. We have so far identified three Wig-1 targets – p53, c-Myc and N-Myc. This target spectrum raises the question why Wig-1 – a p53 target gene and stabilizer of p53 mRNA – positively regulates the Myc oncogenes? Why is Wig-1 fraternizing with the enemy?

We propose three possible models to explain this Wig-1 target selection. First of all, it is possible that Wig-1 has a different set of targets in adult and in embryonic tissues – Wig-1 may regulate p53 in adult cells while the Myc mRNAs may be preferred targets during development. Target specificity may also be deregulated during transformation, rendering the Mycs Wig-1 targets in cancer cells. A different subset of Wig-1 targets in adult and developing tissues could be achieved by selective expression of yet unidentified Wig-1 cofactors. In accordance with a p53 independent role for Wig-1 during embryogenesis, we have shown that basal Wig-1 expression is independent of p53 during embryonic development. In addition, the phenotype of Wig-1 knockout mice differs markedly from that of p53 knockout mice – Wig-1 knockouts are lethal before blastocyst stage while p53 knockouts mainly develop normally[5]. The fact that N-Myc is normally expressed only during embryonic development is also in line with a development-specific subset of Wig-1 targets. Arguing against this theory, however, is the observed Wig-1-mediated regulation of c-Myc in human primary fibroblasts indicating that Wig-1 can regulate c-Myc also in cells that are neither embryonic nor cancerous. See figure 7 a for an illustration.

The second theory is based on a change of Wig-1 target selection after stress. One could imagine that a stress-induced cofactor exchange and/or post-translational modification of Wig-1 could induce target specificity to change from Myc to p53. In accordance with this theory, the Wig-1 protein has several putative phosphorylation sites (Bersani et al, unpublished data), and is acetylated *in vitro* (Mendez-Vidal et al, unpublished data). Also in this scenario, the fact that Wig-1 regulates p53 mRNA through a U-rich and N-Myc through an AU-rich region opens up the possibility of differently regulated Wig-1 specificity and/or different Wig-1 affinity for these two AREs. p53 mRNA is regulated by Wig-1 also in the absence of stress, which speaks against this model. However, it may be possible that Wig-1 always is able to regulate the U-rich region in p53, while it only can regulate the AU-rich region in N-Myc in the absence of stress. See figure 7 b for an illustration.

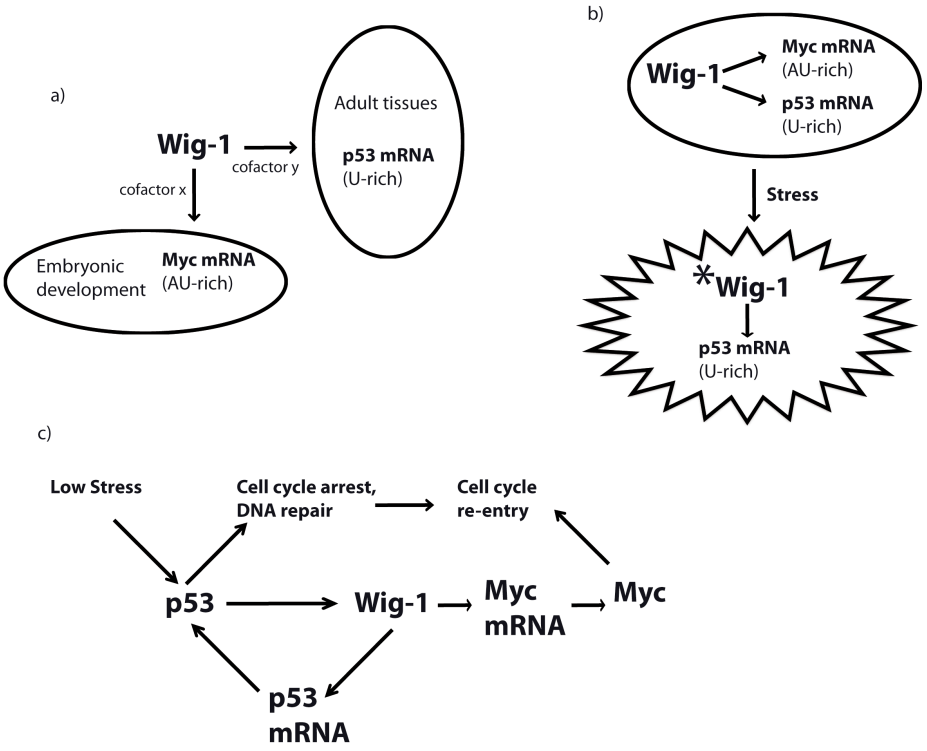

Figure 7: Models for the role of Wig-1 mediated regulation of p53 and Myc. See text for details.

The third possibility is based on the observation that p53 can induce a number of genes that prevent apoptosis and promote survival (reviewed in[63]). Lately, it has been realized that the p53 responses of cell cycle arrest and DNA repair on the one hand, and apoptosis on the other hand, actually can oppose each other. Mild damage induces cell cycle arrest and DNA repair, allowing the cell, once the damage is repaired, to return to a cycling state. In accordance with this, both p21 and 14-3-3σ are anti-apoptotic (reviewed in[63,230]). The perfect consensus p53 response element in the human Wig-1 intron 1 supports a role of Wig-1 in a mild stress response. Perfect consensus p53 response elements are normally found in cell cycle arrest genes such as p21[231]. Additionally, Wig-1 induction after p53 activation has similar kinetics to that of p21[149]. Further support for a role of Wig-1 in an arrest associated response comes form a mouse model knock-in for a p53 mutant only able to activate cell cycle arrest genes. When MEFs from this mouse was treated with γ-radiation, Wig-1 was one of the most strongly upregulated genes[232]. In a mild stress response, where p53 induces arrest and repair followed by return to cycling, it may be beneficial for p53 to also induce a gene – Wig-1 – that will stabilize the Myc mRNAs. This stabilization will provide the cell with a modest increase in factors necessary for proliferation. At the same time, Wig-1 also stabilizes p53 mRNA, thus lowering the threshold for a response to subsequent stress in case of remaining

damage. Further, the low basal levels of p53 have functions differing from those of stress-activated p53 and may actually promote survival (reviewed in[16,63]); hence Wig-1 may be one of these pro-survival targets of basal p53 levels. See figure 7 c for an illustration.

Conclusions in summary

Paper I
Wig-1 binds to RHA and hnRNPA2/B1 through RNA. The RNA binding ability of Wig-1 is important for the biological functions of Wig-1.

Paper II
Wig-1 is an ARE-BP that acts in a positive feedback loop to stabilize p53 mRNA through binding to a U-rich region in the p53 3'UTR.

Paper III
Wig-1 positively regulates N-Myc mRNA through an AU-rich region in the N-Myc 3'UTR, and Wig-1 knockdown induces differentiation in N-Myc amplified neuroblastoma cells.

Paper IV
Wig-1 knockout mice are lethal before the blastocyst stage during embryonic development, possibly because of the simultaneous deregulation of c-Myc and N-Myc.

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References

1 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70, doi:S0092-8674(00)81683-9 [pii] (2000).

2 Lane, D. P. Cancer. p53, guardian of the genome. *Nature* **358**, 15-16, doi:10.1038/358015a0 (1992).

3 Malkin, D. *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**, 1233-1238 (1990).

4 Petitjean, A., Achatz, M. I., Borresen-Dale, A. L., Hainaut, P. & Olivier, M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene* **26**, 2157-2165, doi:1210302 [pii]
10.1038/sj.onc.1210302 (2007).

5 Donehower, L. A. *et al.* Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221, doi:10.1038/356215a0 (1992).

6 Lane, D. P. & Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**, 261-263 (1979).

7 Linzer, D. I. & Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**, 43-52, doi:0092-8674(79)90293-9 [pii] (1979).

8 DeLeo, A. B. *et al.* Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A* **76**, 2420-2424 (1979).

9 Chang, C., Simmons, D. T., Martin, M. A. & Mora, P. T. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. *J Virol* **31**, 463-471 (1979).

10 Oren, M. & Levine, A. J. Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. *Proc Natl Acad Sci U S A* **80**, 56-59 (1983).

11 Zakut-Houri, R. *et al.* A single gene and a pseudogene for the cellular tumour antigen p53. *Nature* **306**, 594-597 (1983).

12 Matlashewski, G. *et al.* Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. *EMBO J* **3**, 3257-3262 (1984).

13 Harris, C. C. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* **17**, 1187-1198 (1996).

14 Baker, S. J. *et al.* Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**, 217-221 (1989).

15 Levine, A. J. & Oren, M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* **9**, 749-758, doi:nrc2723 [pii]
10.1038/nrc2723 (2009).

16 Vousden, K. H. & Prives, C. Blinded by the Light: The Growing Complexity of p53. *Cell* **137**, 413-431, doi:S0092-8674(09)00459-0 [pii]
10.1016/j.cell.2009.04.037 (2009).

17 Vousden, K. H. & Ryan, K. M. p53 and metabolism. *Nat Rev Cancer* **9**, 691-700, doi:nrc2715 [pii]
10.1038/nrc2715 (2009).

18 Zhang, Y. & Lu, H. Signaling to p53: ribosomal proteins find their way. *Cancer Cell* **16**, 369-377, doi:S1535-6108(09)00335-3 [pii]
10.1016/j.ccr.2009.09.024 (2009).

19 Bartek, J. & Lukas, J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**, 421-429, doi:S1535610803001107 [pii] (2003).

20 Bates, S. *et al.* p14ARF links the tumour suppressors RB and p53. *Nature* **395**, 124-125, doi:10.1038/25867 (1998).
21 Pomerantz, J. *et al.* The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92**, 713-723, doi:S0092-8674(00)81400-2 [pii] (1998).
22 Zhang, Y., Xiong, Y. & Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92**, 725-734, doi:S0092-8674(00)81401-4 [pii] (1998).
23 Gallagher, S. J., Kefford, R. F. & Rizos, H. The ARF tumour suppressor. *Int J Biochem Cell Biol* **38**, 1637-1641, doi:S1357-2725(06)00068-9 [pii]
10.1016/j.biocel.2006.02.008 (2006).
24 Gorgoulis, V. G. *et al.* Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**, 907-913, doi:nature03485 [pii]
10.1038/nature03485 (2005).
25 Bartkova, J. *et al.* DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434**, 864-870, doi:nature03482 [pii]
10.1038/nature03482 (2005).
26 Vousden, K. H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604, doi:10.1038/nrc864
nrc864 [pii] (2002).
27 Resnick-Silverman, L. & Manfredi, J. J. Gene-specific mechanisms of p53 transcriptional control and prospects for cancer therapy. *J Cell Biochem* **99**, 679-689, doi:10.1002/jcb.20925 (2006).
28 Menendez, D., Inga, A. & Resnick, M. A. The expanding universe of p53 targets. *Nat Rev Cancer* **9**, 724-737, doi:nrc2730 [pii]
10.1038/nrc2730 (2009).
29 Thut, C. J., Chen, J. L., Klemm, R. & Tjian, R. p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science* **267**, 100-104 (1995).
30 Farmer, G., Colgan, J., Nakatani, Y., Manley, J. L. & Prives, C. Functional interaction between p53, the TATA-binding protein (TBP), andTBP-associated factors in vivo. *Mol Cell Biol* **16**, 4295-4304 (1996).
31 Riley, T., Sontag, E., Chen, P. & Levine, A. Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* **9**, 402-412, doi:nrm2395 [pii]
10.1038/nrm2395 (2008).
32 Miyashita, T., Harigai, M., Hanada, M. & Reed, J. C. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* **54**, 3131-3135 (1994).
33 Wang, B., Xiao, Z., Ko, H. L. & Ren, E. C. The p53 response element and transcriptional repression. *Cell Cycle* **9**, 870-879, doi:10825 [pii] (2010).
34 Marchenko, N. D., Zaika, A. & Moll, U. M. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem* **275**, 16202-16212, doi:275/21/16202 [pii] (2000).
35 Speidel, D. Transcription-independent p53 apoptosis: an alternative route to death. *Trends Cell Biol* **20**, 14-24, doi:S0962-8924(09)00240-2 [pii]
10.1016/j.tcb.2009.10.002 (2010).
36 Suzuki, H. I. *et al.* Modulation of microRNA processing by p53. *Nature* **460**, 529-533 (2009).
37 el-Deiry, W. S. *et al.* WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-825, doi:0092-8674(93)90500-P [pii] (1993).

38 Hermeking, H. *et al.* 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* **1**, 3-11, doi:S1097-2765(00)80002-7 [pii] (1997).
39 Sionov, R. V. & Haupt, Y. The cellular response to p53: the decision between life and death. *Oncogene* **18**, 6145-6157, doi:10.1038/sj.onc.1203130 (1999).
40 Abbas, T. & Dutta, A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* **9**, 400-414, doi:nrc2657 [pii]
10.1038/nrc2657 (2009).
41 Taylor, W. R. & Stark, G. R. Regulation of the G2/M transition by p53. *Oncogene* **20**, 1803-1815, doi:10.1038/sj.onc.1204252 (2001).
42 Muller, M. *et al.* p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* **188**, 2033-2045 (1998).
43 Miyashita, T. & Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-299, doi:0092-8674(95)90412-3 [pii] (1995).
44 Nakano, K. & Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**, 683-694, doi:S1097-2765(01)00214-3 [pii] (2001).
45 Oda, E. *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053-1058, doi:8508 [pii] (2000).
46 Creagh, E. M., Conroy, H. & Martin, S. J. Caspase-activation pathways in apoptosis and immunity. *Immunol Rev* **193**, 10-21, doi:048 [pii] (2003).
47 Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**, 675-684, doi:0092-8674(95)90039-X [pii] (1995).
48 Liu, G. *et al.* Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. *Nat Genet* **36**, 63-68, doi:10.1038/ng1282
ng1282 [pii] (2004).
49 Evan, G. I. & d'Adda di Fagagna, F. Cellular senescence: hot or what? *Curr Opin Genet Dev* **19**, 25-31, doi:S0959-437X(08)00191-3 [pii]
10.1016/j.gde.2008.11.009 (2009).
50 Helton, E. S. & Chen, X. p53 modulation of the DNA damage response. *J Cell Biochem* **100**, 883-896, doi:10.1002/jcb.21091 (2007).
51 Zuckerman, V., Wolyniec, K., Sionov, R. V., Haupt, S. & Haupt, Y. Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol* **219**, 3-15, doi:10.1002/path.2584 (2009).
52 Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**, 725-730, doi:nature03918 [pii]
10.1038/nature03918 (2005).
53 Cicalese, A. *et al.* The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* **138**, 1083-1095, doi:S0092-8674(09)00840-X [pii]
10.1016/j.cell.2009.06.048 (2009).
54 Zhao, T. & Xu, Y. p53 and stem cells: new developments and new concerns. *Trends Cell Biol* **20**, 170-175, doi:S0962-8924(09)00299-2 [pii]
10.1016/j.tcb.2009.12.004 (2010).
55 Song, H., Chung, S. K. & Xu, Y. Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. *Cell Stem Cell* **6**, 80-89, doi:S1934-5909(09)00623-7 [pii]
10.1016/j.stem.2009.11.016 (2010).
56 Kawamura, T. *et al.* Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* **460**, 1140-1144, doi:nature08311 [pii]

10.1038/nature08311 (2009).
57 Armstrong, J. F., Kaufman, M. H., Harrison, D. J. & Clarke, A. R. High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* **5**, 931-936, doi:S0960-9822(95)00183-7 [pii] (1995).
58 Sah, V. P. *et al.* A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* **10**, 175-180, doi:10.1038/ng0695-175 (1995).
59 Hu, W., Feng, Z., Teresky, A. K. & Levine, A. J. p53 regulates maternal reproduction through LIF. *Nature* **450**, 721-724, doi:nature05993 [pii]
10.1038/nature05993 (2007).
60 Smith, M. L. *et al.* p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Mol Cell Biol* **20**, 3705-3714 (2000).
61 Gatz, S. A. & Wiesmuller, L. p53 in recombination and repair. *Cell Death Differ* **13**, 1003-1016, doi:4401903 [pii]
10.1038/sj.cdd.4401903 (2006).
62 Dameron, K. M., Volpert, O. V., Tainsky, M. A. & Bouck, N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* **265**, 1582-1584 (1994).
63 Janicke, R. U., Sohn, D. & Schulze-Osthoff, K. The dark side of a tumor suppressor: anti-apoptotic p53. *Cell Death Differ* **15**, 959-976, doi:cdd200833 [pii]
10.1038/cdd.2008.33 (2008).
64 Tyner, S. D. *et al.* p53 mutant mice that display early ageing-associated phenotypes. *Nature* **415**, 45-53, doi:10.1038/415045a
415045a [pii] (2002).
65 Garcia-Cao, I. *et al.* "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. *EMBO J* **21**, 6225-6235 (2002).
66 Matheu, A., Maraver, A. & Serrano, M. The Arf/p53 pathway in cancer and aging. *Cancer Res* **68**, 6031-6034, doi:68/15/6031 [pii]
10.1158/0008-5472.CAN-07-6851 (2008).
67 Junttila, M. R. & Evan, G. I. p53--a Jack of all trades but master of none. *Nat Rev Cancer* **9**, 821-829, doi:nrc2728 [pii]
10.1038/nrc2728 (2009).
68 Collavin, L., Lunardi, A. & Del Sal, G. p53-family proteins and their regulators: hubs and spokes in tumor suppression. *Cell Death Differ* **17**, 901-911, doi:cdd201035 [pii]
10.1038/cdd.2010.35 (2010).
69 Pluta, A. *et al.* The role of p73 in hematological malignancies. *Leukemia* **20**, 757-766, doi:2404166 [pii]
10.1038/sj.leu.2404166 (2006).
70 Mills, A. A. *et al.* p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713, doi:10.1038/19531 (1999).
71 Yang, A. *et al.* p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714-718, doi:10.1038/19539 (1999).
72 Yang, A. *et al.* p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404**, 99-103, doi:10.1038/35003607 (2000).
73 Momand, J., Zambetti, G. P., Olson, D. C., George, D. & Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237-1245, doi:0092-8674(92)90644-R [pii] (1992).
74 Kubbutat, M. H., Jones, S. N. & Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature* **387**, 299-303, doi:10.1038/387299a0 (1997).

75 Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature* **387**, 296-299, doi:10.1038/387296a0 (1997).
76 Honda, R., Tanaka, H. & Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420**, 25-27, doi:S0014-5793(97)01480-4 [pii] (1997).
77 Wu, X., Bayle, J. H., Olson, D. & Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* **7**, 1126-1132 (1993).
78 Marine, J. C. & Lozano, G. Mdm2-mediated ubiquitylation: p53 and beyond. *Cell Death Differ* **17**, 93-102, doi:cdd200968 [pii]
10.1038/cdd.2009.68 (2010).
79 Kruse, J. P. & Gu, W. Modes of p53 regulation. *Cell* **137**, 609-622, doi:S0092-8674(09)00511-X [pii]
10.1016/j.cell.2009.04.050 (2009).
80 Montes de Oca Luna, R., Wagner, D. S. & Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378**, 203-206, doi:10.1038/378203a0 (1995).
81 Jones, S. N., Roe, A. E., Donehower, L. A. & Bradley, A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378**, 206-208, doi:10.1038/378206a0 (1995).
82 Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L. & Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* **358**, 80-83, doi:10.1038/358080a0 (1992).
83 Marine, J. C. & Jochemsen, A. G. Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun* **331**, 750-760, doi:S0006-291X(05)00657-1 [pii]
10.1016/j.bbrc.2005.03.151 (2005).
84 Liu, H., Lu, Z. G., Miki, Y. & Yoshida, K. Protein kinase C delta induces transcription of the TP53 tumor suppressor gene by controlling death-promoting factor Btf in the apoptotic response to DNA damage. *Molecular and cellular biology* **27**, 8480-8491 (2007).
85 Raman, V. *et al.* Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature* **405**, 974-978 (2000).
86 Phan, R. T. & Dalla-Favera, R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432**, 635-639, doi:nature03147 [pii]
10.1038/nature03147 (2004).
87 Lynch, C. J. & Milner, J. Loss of one p53 allele results in four-fold reduction of p53 mRNA and protein: a basis for p53 haplo-insufficiency. *Oncogene* **25**, 3463-3470 (2006).
88 Muhlrad, D. & Parker, R. Premature translational termination triggers mRNA decapping. *Nature* **370**, 578-581, doi:10.1038/370578a0 (1994).
89 Holcik, M. & Sonenberg, N. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* **6**, 318-327, doi:nrm1618 [pii]
10.1038/nrm1618 (2005).
90 Brawerman, G. The Role of the poly(A) sequence in mammalian messenger RNA. *CRC Crit Rev Biochem* **10**, 1-38 (1981).
91 Millevoi, S. & Vagner, S. Molecular mechanisms of eukaryotic pre-mRNA 3' end processing regulation. *Nucleic Acids Res* **38**, 2757-2774, doi:gkp1176 [pii]
10.1093/nar/gkp1176 (2010).
92 Mangus, D. A., Evans, M. C. & Jacobson, A. Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biol* **4**, 223, doi:10.1186/gb-2003-4-7-223 (2003).

93 Meyer, S., Temme, C. & Wahle, E. Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol Biol* **39**, 197-216, doi:39/4/197 [pii]
10.1080/10409230490513991 (2004).
94 Barreau, C., Paillard, L. & Osborne, H. B. AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res* **33**, 7138-7150, doi:33/22/7138 [pii]
10.1093/nar/gki1012 (2005).
95 Audic, Y. & Hartley, R. S. Post-transcriptional regulation in cancer. *Biol Cell* **96**, 479-498, doi:10.1016/j.biolcel.2004.05.002
S0248490004001108 [pii] (2004).
96 von Roretz, C. & Gallouzi, I. E. Decoding ARE-mediated decay: is microRNA part of the equation? *J Cell Biol* **181**, 189-194, doi:jcb.200712054 [pii]
10.1083/jcb.200712054 (2008).
97 Steinman, R. A. mRNA stability control: a clandestine force in normal and malignant hematopoiesis. *Leukemia* **21**, 1158-1171, doi:2404656 [pii]
10.1038/sj.leu.2404656 (2007).
98 Mazan-Mamczarz, K. *et al.* RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. *Proc Natl Acad Sci U S A* **100**, 8354-8359, doi:10.1073/pnas.1432104100
1432104100 [pii] (2003).
99 Galban, S. *et al.* Influence of the RNA-binding protein HuR in pVHL-regulated p53 expression in renal carcinoma cells. *Mol Cell Biol* **23**, 7083-7095 (2003).
100 Fu, L., Ma, W. & Benchimol, S. A translation repressor element resides in the 3' untranslated region of human p53 mRNA. *Oncogene* **18**, 6419-6424 (1999).
101 Schumacher, B. *et al.* Translational repression of C. elegans p53 by GLD-1 regulates DNA damage-induced apoptosis. *Cell* **120**, 357-368 (2005).
102 Zou, T. *et al.* Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. *J Biol Chem* **281**, 19387-19394, doi:M602344200 [pii]
10.1074/jbc.M602344200 (2006).
103 Ghosh, M. *et al.* Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *The Journal of clinical investigation* **119**, 3530-3543 (2009).
104 Nabors, L. B., Gillespie, G. Y., Harkins, L. & King, P. H. HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs. *Cancer research* **61**, 2154-2161 (2001).
105 Brosens, L. A. *et al.* Increased expression of cytoplasmic HuR in familial adenomatous polyposis. *Cancer biology & therapy* **7**, 424-427 (2008).
106 Denkert, C. *et al.* Expression of the ELAV-like protein HuR in human colon cancer: association with tumor stage and cyclooxygenase-2. *Mod Pathol* **19**, 1261-1269 (2006).
107 Denkert, C. *et al.* Expression of the ELAV-like protein HuR is associated with higher tumor grade and increased cyclooxygenase-2 expression in human breast carcinoma. *Clin Cancer Res* **10**, 5580-5586 (2004).
108 Stoecklin, G., Stoeckle, P., Lu, M., Muehlemann, O. & Moroni, C. Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. *RNA (New York, N.Y* **7**, 1578-1588 (2001).
109 Brennan, S. E. *et al.* The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. *Cancer research* **69**, 5168-5176 (2009).

110 Yang, X. *et al.* Prostaglandin A2-mediated stabilization of p21 mRNA through an ERK-dependent pathway requiring the RNA-binding protein HuR. *J Biol Chem* **279**, 49298-49306, doi:10.1074/jbc.M407535200
M407535200 [pii] (2004).
111 Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811, doi:10.1038/35888 (1998).
112 Brodersen, P. & Voinnet, O. Revisiting the principles of microRNA target recognition and mode of action. *Nature reviews* **10**, 141-148 (2009).
113 Ghildiyal, M. & Zamore, P. D. Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10**, 94-108 (2009).
114 Agami, R. microRNAs, RNA binding proteins and cancer. *Eur J Clin Invest* **40**, 370-374, doi:ECI2279 [pii]
10.1111/j.1365-2362.2010.02279.x (2010).
115 Le, M. T. *et al.* MicroRNA-125b is a novel negative regulator of p53. *Genes & development* **23**, 862-876 (2009).
116 Zhang, Y. *et al.* MicroRNA 125a and its regulation of the p53 tumor suppressor gene. *FEBS letters* **583**, 3725-3730 (2009).
117 Hu, W. *et al.* Negative regulation of tumor suppressor p53 by microRNA miR-504. *Mol Cell* **38**, 689-699, doi:S1097-2765(10)00413-2 [pii]
10.1016/j.molcel.2010.05.027 (2010).
118 Li, J. *et al.* miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. *PLoS Genet* **6**, e1000795, doi:10.1371/journal.pgen.1000795 (2010).
119 Tian, S. *et al.* MicroRNA-1285 inhibits the expression of p53 by directly targeting its 3' untranslated region. *Biochem Biophys Res Commun* **396**, 435-439, doi:S0006-291X(10)00792-8 [pii]
10.1016/j.bbrc.2010.04.112 (2010).
120 Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-838 (2005).
121 Kumar, M. S., Lu, J., Mercer, K. L., Golub, T. R. & Jacks, T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nature genetics* **39**, 673-677 (2007).
122 Hausser, J., Landthaler, M., Jaskiewicz, L., Gaidatzis, D. & Zavolan, M. Relative contribution of sequence and structure features to the mRNA binding of Argonaute/EIF2C-miRNA complexes and the degradation of miRNA targets. *Genome research* **19**, 2009-2020 (2009).
123 Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Molecular cell* **27**, 91-105, doi:S1097-2765(07)00407-8 [pii]
10.1016/j.molcel.2007.06.017 (2007).
124 Vasudevan, S. & Steitz, J. A. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell* **128**, 1105-1118, doi:S0092-8674(07)00201-2 [pii]
10.1016/j.cell.2007.01.038 (2007).
125 Kedde, M. *et al.* RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* **131**, 1273-1286, doi:S0092-8674(07)01537-1 [pii]
10.1016/j.cell.2007.11.034 (2007).
126 Kim, H. H. *et al.* HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev* **23**, 1743-1748, doi:gad.1812509 [pii]
10.1101/gad.1812509 (2009).

127 Katsanou, V. *et al.* The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development. *Mol Cell Biol* **29**, 2762-2776, doi:MCB.01393-08 [pii]
10.1128/MCB.01393-08 (2009).
128 de Vries, W. N. *et al.* Reprogramming and differentiation in mammals: motifs and mechanisms. *Cold Spring Harb Symp Quant Biol* **73**, 33-38, doi:sqb.2008.73.016 [pii]
10.1101/sqb.2008.73.016 (2008).
129 Bernstein, E. *et al.* Dicer is essential for mouse development. *Nat Genet* **35**, 215-217, doi:10.1038/ng1253
ng1253 [pii] (2003).
130 Wang, Y., Medvid, R., Melton, C., Jaenisch, R. & Blelloch, R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* **39**, 380-385, doi:ng1969 [pii]
10.1038/ng1969 (2007).
131 Ventura, A. *et al.* Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132**, 875-886, doi:S0092-8674(08)00267-5 [pii]
10.1016/j.cell.2008.02.019 (2008).
132 Kedde, M. & Agami, R. Interplay between microRNAs and RNA-binding proteins determines developmental processes. *Cell Cycle* **7**, 899-903, doi:5644 [pii] (2008).
133 Grover, R., Candeias, M. M., Fahraeus, R. & Das, S. p53 and little brother p53/47: linking IRES activities with protein functions. *Oncogene* **28**, 2766-2772 (2009).
134 Takagi, M., Absalon, M. J., McLure, K. G. & Kastan, M. B. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* **123**, 49-63 (2005).
135 Mahmoudi, S. *et al.* Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. *Molecular cell* **33**, 462-471 (2009).
136 Candeias, M. M. *et al.* p53 mRNA controls p53 activity by managing Mdm2 functions. *Nature cell biology* (2008).
137 Bommer, G. T. *et al.* p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* **17**, 1298-1307 (2007).
138 Raver-Shapira, N. *et al.* Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Molecular cell* **26**, 731-743 (2007).
139 Tarasov, V. *et al.* Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell cycle (Georgetown, Tex* **6**, 1586-1593 (2007).
140 He, L. *et al.* A microRNA component of the p53 tumour suppressor network. *Nature* **447**, 1130-1134 (2007).
141 Corney, D. C., Flesken-Nikitin, A., Godwin, A. K., Wang, W. & Nikitin, A. Y. MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer research* **67**, 8433-8438 (2007).
142 Sachdeva, M. *et al.* p53 represses c-Myc through induction of the tumor suppressor miR-145. *Proc Natl Acad Sci U S A* **106**, 3207-3212, doi:0808042106 [pii]
10.1073/pnas.0808042106 (2009).
143 Yan, H. L. *et al.* Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. *The EMBO journal* **28**, 2719-2732 (2009).
144 Mudhasani, R. *et al.* Loss of miRNA biogenesis induces p19Arf-p53 signaling and senescence in primary cells. *The Journal of cell biology* **181**, 1055-1063 (2008).

145 Mosner, J. *et al.* Negative feedback regulation of wild-type p53 biosynthesis. *The EMBO journal* **14**, 4442-4449 (1995).
146 Miller, S. J., Suthiphongchai, T., Zambetti, G. P. & Ewen, M. E. p53 binds selectively to the 5' untranslated region of cdk4, an RNA element necessary and sufficient for transforming growth factor beta- and p53-mediated translational inhibition of cdk4. *Molecular and cellular biology* **20**, 8420-8431 (2000).
147 Gonzalez-Herrera, I. G. *et al.* IRES-dependent regulation of FGF-2 mRNA translation in pathophysiological conditions in the mouse. *Biochemical Society transactions* **34**, 17-21 (2006).
148 Galy, B., Creancier, L., Prado-Lourenco, L., Prats, A. C. & Prats, H. p53 directs conformational change and translation initiation blockade of human fibroblast growth factor 2 mRNA. *Oncogene* **20**, 4613-4620 (2001).
149 Varmeh-Ziaie, S. *et al.* Wig-1, a new p53-induced gene encoding a zinc finger protein. *Oncogene* **15**, 2699-2704, doi:10.1038/sj.onc.1201454 (1997).
150 Hellborg, F. *et al.* Human wig-1, a p53 target gene that encodes a growth inhibitory zinc finger protein. *Oncogene* **20**, 5466-5474, doi:10.1038/sj.onc.1204722 (2001).
151 Israeli, D. *et al.* A novel p53-inducible gene, PAG608, encodes a nuclear zinc finger protein whose overexpression promotes apoptosis. *EMBO J* **16**, 4384-4392, doi:10.1093/emboj/16.14.4384 (1997).
152 Hellborg, F. & Wiman, K. G. The p53-induced Wig-1 zinc finger protein is highly conserved from fish to man. *Int J Oncol* **24**, 1559-1564 (2004).
153 Wilhelm, M. T., Mendez-Vidal, C. & Wiman, K. G. Identification of functional p53-binding motifs in the mouse wig-1 promoter. *FEBS Lett* **524**, 69-72, doi:S0014579302030041 [pii] (2002).
154 Chen, T., Brownawell, A. M. & Macara, I. G. Nucleocytoplasmic shuttling of JAZ, a new cargo protein for exportin-5. *Molecular and cellular biology* **24**, 6608-6619 (2004).
155 Yang, M., Wu, S., Su, X. & May, W. S. JAZ mediates G1 cell-cycle arrest and apoptosis by positively regulating p53 transcriptional activity. *Blood* **108**, 4136-4145 (2006).
156 Mendez-Vidal, C., Wilhelm, M. T., Hellborg, F., Qian, W. & Wiman, K. G. The p53-induced mouse zinc finger protein wig-1 binds double-stranded RNA with high affinity. *Nucleic Acids Res* **30**, 1991-1996 (2002).
157 Mendez Vidal, C., Prahl, M. & Wiman, K. G. The p53-induced Wig-1 protein binds double-stranded RNAs with structural characteristics of siRNAs and miRNAs. *FEBS Lett* **580**, 4401-4408, doi:S0014-5793(06)00838-6 [pii]
10.1016/j.febslet.2006.07.005 (2006).
158 Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. & Melton, D. A. "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298**, 597-600, doi:10.1126/science.1072530
1072530 [pii] (2002).
159 Park, I. K. *et al.* Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302-305, doi:10.1038/nature01587
nature01587 [pii] (2003).
160 Gillardon, F., Spranger, M., Tiesler, C. & Hossmann, K. A. Expression of cell death-associated phospho-c-Jun and p53-activated gene 608 in hippocampal CA1 neurons following global ischemia. *Brain Res Mol Brain Res* **73**, 138-143. (1999).
161 Tomasevic, G., Shamloo, M., Israeli, D. & Wieloch, T. Activation of p53 and its target genes p21(WAF1/Cip1) and PAG608/Wig-1 in ischemic preconditioning. *Brain Res Mol Brain Res* **70**, 304-313. (1999).

162 Asanuma, M. *et al.* Suppression of p53-activated gene, PAG608, attenuates methamphetamine-induced neurotoxicity. *Neurosci Lett* **414**, 263-267, doi:S0304-3940(06)01350-4 [pii]
10.1016/j.neulet.2006.12.036 (2007).
163 Morimoto, N. *et al.* Induction of parkinsonism-related proteins in the spinal motor neurons of transgenic mouse carrying a mutant SOD1 gene. *J Neurosci Res* **88**, 1804-1811, doi:10.1002/jnr.22341 (2010).
164 Shimizu, M. *et al.* Specific induction of PAG608 in cranial and spinal motor neurons of L-DOPA-treated parkinsonian rats. *Neurosci Res* **60**, 355-363, doi:S0168-0102(07)01859-7 [pii]
10.1016/j.neures.2007.12.006 (2008).
165 Higashi, Y. *et al.* The p53-activated gene, PAG608, requires a zinc finger domain for nuclear localization and oxidative stress-induced apoptosis. *J Biol Chem* **277**, 42224-42232, doi:10.1074/jbc.M203594200
M203594200 [pii] (2002).
166 Nakajima, T. *et al.* RNA helicase A mediates association of CBP with RNA polymerase II. *Cell* **90**, 1107-1112 (1997).
167 Anderson, S. F., Schlegel, B. P., Nakajima, T., Wolpin, E. S. & Parvin, J. D. BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Nature genetics* **19**, 254-256 (1998).
168 Tetsuka, T. *et al.* RNA helicase A interacts with nuclear factor kappaB p65 and functions as a transcriptional coactivator. *European Journal of Biochemistry* **271**, 3741-3751 (2004).
169 Zhang, S. & Grosse, F. Multiple functions of nuclear DNA helicase II (RNA helicase A) in nucleic acid metabolism. *Acta Biochim Biophys Sin (Shanghai)* **36**, 177-183 (2004).
170 Hartman, T. R. *et al.* RNA helicase A is necessary for translation of selected messenger RNAs. *Nature structural & molecular biology* **13**, 509-516 (2006).
171 Kozu, T., Henrich, B. & Schafer, K. P. Structure and expression of the gene (HNRPA2B1) encoding the human hnRNP protein A2/B1. *Genomics* **25**, 365-371, doi:0888-7543(95)80035-K [pii] (1995).
172 He, Y. & Smith, R. Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. *Cell Mol Life Sci* **66**, 1239-1256, doi:10.1007/s00018-008-8532-1 (2009).
173 Hamilton, B. J. *et al.* hnRNP A2 and hnRNP L bind the 3'UTR of glucose transporter 1 mRNA and exist as a complex in vivo. *Biochem Biophys Res Commun* **261**, 646-651, doi:10.1006/bbrc.1999.1040
S0006-291X(99)91040-9 [pii] (1999).
174 Fahling, M. *et al.* Heterogeneous nuclear ribonucleoprotein-A2/B1 modulate collagen prolyl 4-hydroxylase, alpha (I) mRNA stability. *J Biol Chem* **281**, 9279-9286, doi:M510925200 [pii]
10.1074/jbc.M510925200 (2006).
175 Meyer, N. & Penn, L. Z. Reflecting on 25 years with MYC. *Nat Rev Cancer* **8**, 976-990, doi:nrc2231 [pii]
10.1038/nrc2231 (2008).
176 Schwab, M. *et al.* Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* **305**, 245-248 (1983).
177 Kohl, N. E. *et al.* Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* **35**, 359-367, doi:0092-8674(83)90169-1 [pii] (1983).

178 Nau, M. M. *et al.* L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* **318**, 69-73 (1985).
179 Soucek, L. & Evan, G. I. The ups and downs of Myc biology. *Curr Opin Genet Dev* **20**, 91-95, doi:S0959-437X(09)00177-4 [pii]
10.1016/j.gde.2009.11.001 (2010).
180 Eilers, M. & Eisenman, R. N. Myc's broad reach. *Genes Dev* **22**, 2755-2766, doi:22/20/2755 [pii]
10.1101/gad.1712408 (2008).
181 Larsson, L. G. & Henriksson, M. A. The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. *Exp Cell Res* **316**, 1429-1437, doi:S0014-4827(10)00145-X [pii]
10.1016/j.yexcr.2010.03.025 (2010).
182 Fernandez, P. C. *et al.* Genomic targets of the human c-Myc protein. *Genes Dev* **17**, 1115-1129, doi:10.1101/gad.1067003
U-10670R [pii] (2003).
183 Li, Z. *et al.* A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci U S A* **100**, 8164-8169, doi:10.1073/pnas.1332764100
1332764100 [pii] (2003).
184 Roussel, M. F., Cleveland, J. L., Shurtleff, S. A. & Sherr, C. J. Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling. *Nature* **353**, 361-363, doi:10.1038/353361a0 (1991).
185 Karn, J., Watson, J. V., Lowe, A. D., Green, S. M. & Vedeckis, W. Regulation of cell cycle duration by c-myc levels. *Oncogene* **4**, 773-787 (1989).
186 Arabi, A. *et al.* c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat Cell Biol* **7**, 303-310, doi:ncb1225 [pii]
10.1038/ncb1225 (2005).
187 Zindy, F. *et al.* Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* **12**, 2424-2433 (1998).
188 Murphy, D. J. *et al.* Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell* **14**, 447-457, doi:S1535-6108(08)00367-X [pii]
10.1016/j.ccr.2008.10.018 (2008).
189 Dean, M. *et al.* Regulation of c-myc transcription and mRNA abundance by serum growth factors and cell contact. *J Biol Chem* **261**, 9161-9166 (1986).
190 King, P. H. RNA-binding analyses of HuC and HuD with the VEGF and c-myc 3'-untranslated regions using a novel ELISA-based assay. *Nucleic Acids Res* **28**, E20, doi:gnd020 [pii] (2000).
191 Ma, W. J., Cheng, S., Campbell, C., Wright, A. & Furneaux, H. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J Biol Chem* **271**, 8144-8151 (1996).
192 Manohar, C. F. *et al.* HuD, a neuronal-specific RNA-binding protein, increases the in vivo stability of MYCN RNA. *J Biol Chem* **277**, 1967-1973, doi:10.1074/jbc.M106966200
M106966200 [pii] (2002).
193 Ross, R. A. *et al.* HuD, a neuronal-specific RNA-binding protein, is a potential regulator of MYCN expression in human neuroblastoma cells. *Eur J Cancer* **33**, 2071-2074, doi:S0959-8049(97)00331-6 [pii] (1997).
194 Jones, T. R. & Cole, M. D. Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. *Mol Cell Biol* **7**, 4513-4521 (1987).
195 Liao, B., Hu, Y. & Brewer, G. Competitive binding of AUF1 and TIAR to MYC mRNA controls its translation. *Nat Struct Mol Biol* **14**, 511-518, doi:nsmb1249 [pii]

10.1038/nsmb1249 (2007).
196 Cannell, I. G. *et al.* p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. *Proc Natl Acad Sci U S A* **107**, 5375-5380, doi:0910015107 [pii]
10.1073/pnas.0910015107 (2010).
197 Wei, J. S. *et al.* The MYCN oncogene is a direct target of miR-34a. *Oncogene* **27**, 5204-5213, doi:onc2008154 [pii]
10.1038/onc.2008.154 (2008).
198 Lal, A. *et al.* miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR microRNA recognition elements. *Mol Cell* **35**, 610-625, doi:S1097-2765(09)00600-5 [pii]
10.1016/j.molcel.2009.08.020 (2009).
199 Bernstein, P. L., Herrick, D. J., Prokipcak, R. D. & Ross, J. Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. *Genes Dev* **6**, 642-654 (1992).
200 Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R. & Bradley, A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev* **7**, 671-682 (1993).
201 Stanton, B. R., Perkins, A. S., Tessarollo, L., Sassoon, D. A. & Parada, L. F. Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev* **6**, 2235-2247 (1992).
202 Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L. & Rossant, J. A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev* **6**, 691-704 (1992).
203 Charron, J. *et al.* Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. *Genes Dev* **6**, 2248-2257 (1992).
204 Sawai, S., Shimono, A., Hanaoka, K. & Kondoh, H. Embryonic lethality resulting from disruption of both N-myc alleles in mouse zygotes. *New Biol* **3**, 861-869 (1991).
205 Hatton, K. S. *et al.* Expression and activity of L-Myc in normal mouse development. *Mol Cell Biol* **16**, 1794-1804 (1996).
206 Knoepfler, P. S., Cheng, P. F. & Eisenman, R. N. N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev* **16**, 2699-2712, doi:10.1101/gad.1021202 (2002).
207 Douglas, N. C., Jacobs, H., Bothwell, A. L. & Hayday, A. C. Defining the specific physiological requirements for c-Myc in T cell development. *Nat Immunol* **2**, 307-315, doi:10.1038/86308 (2001).
208 Laurenti, E. *et al.* Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. *Cell Stem Cell* **3**, 611-624, doi:S1934-5909(08)00458-X [pii]
10.1016/j.stem.2008.09.005 (2008).
209 Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676, doi:S0092-8674(06)00976-7 [pii]
10.1016/j.cell.2006.07.024 (2006).
210 Varlakhanova, N. V. *et al.* myc maintains embryonic stem cell pluripotency and self-renewal. *Differentiation*, doi:S0301-4681(10)00048-4 [pii]
10.1016/j.diff.2010.05.001 (2010).
211 Laurenti, E., Wilson, A. & Trumpp, A. Myc's other life: stem cells and beyond. *Curr Opin Cell Biol* **21**, 844-854, doi:S0955-0674(09)00170-7 [pii]
10.1016/j.ceb.2009.09.006 (2009).

212 Maris, J. M., Hogarty, M. D., Bagatell, R. & Cohn, S. L. Neuroblastoma. *Lancet* **369**, 2106-2120, doi:S0140-6736(07)60983-0 [pii]
10.1016/S0140-6736(07)60983-0 (2007).
213 Edsjo, A., Holmquist, L. & Pahlman, S. Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell dedifferentiation. *Semin Cancer Biol* **17**, 248-256, doi:S1044-579X(06)00037-X [pii]
10.1016/j.semcancer.2006.04.005 (2007).
214 Johnsen, J. I., Kogner, P., Albihn, A. & Henriksson, M. A. Embryonal neural tumours and cell death. *Apoptosis* **14**, 424-438, doi:10.1007/s10495-009-0325-y (2009).
215 Schmidt, M. L. *et al.* Biologic factors determine prognosis in infants with stage IV neuroblastoma: A prospective Children's Cancer Group study. *J Clin Oncol* **18**, 1260-1268 (2000).
216 Weiss, W. A., Aldape, K., Mohapatra, G., Feuerstein, B. G. & Bishop, J. M. Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J* **16**, 2985-2995, doi:10.1093/emboj/16.11.2985 (1997).
217 Kang, J. H. *et al.* MYCN silencing induces differentiation and apoptosis in human neuroblastoma cells. *Biochem Biophys Res Commun* **351**, 192-197, doi:S0006-291X(06)02251-0 [pii]
10.1016/j.bbrc.2006.10.020 (2006).
218 He, Y., Brown, M. A., Rothnagel, J. A., Saunders, N. A. & Smith, R. Roles of heterogeneous nuclear ribonucleoproteins A and B in cell proliferation. *J Cell Sci* **118**, 3173-3183, doi:118/14/3173 [pii]
10.1242/jcs.02448 (2005).
219 Pahlman, S., Meyerson, G., Lindgren, E., Schalling, M. & Johansson, I. Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proc Natl Acad Sci U S A* **88**, 9994-9998 (1991).
220 Guo, G. *et al.* Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. *Dev Cell* **18**, 675-685, doi:S1534-5807(10)00110-3 [pii]
10.1016/j.devcel.2010.02.012 (2010).
221 Malynn, B. A. *et al.* N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. *Genes Dev* **14**, 1390-1399 (2000).
222 Shen-Li, H. *et al.* Essential role for Max in early embryonic growth and development. *Genes Dev* **14**, 17-22 (2000).
223 Langa, F. *et al.* Healthy mice with an altered c-myc gene: role of the 3' untranslated region revisited. *Oncogene* **20**, 4344-4353, doi:10.1038/sj.onc.1204482 (2001).
224 Uetani, N., Yamaura, K. & Sato, K. Expression in situ of c-myc mRNA and c-myc protein during spermatogenesis in the adult mouse. *Cell Biol Int* **18**, 85-87, doi:S1065-6995(84)71046-8 [pii]
10.1006/cbir.1994.1046 (1994).
225 Braydich-Stolle, L., Kostereva, N., Dym, M. & Hofmann, M. C. Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. *Dev Biol* **304**, 34-45, doi:S0012-1606(06)01431-X [pii]
10.1016/j.ydbio.2006.12.013 (2007).
226 Suzuki, T., Abe, K., Inoue, A. & Aoki, F. Expression of c-MYC in nuclear speckles during mouse oocyte growth and preimplantation development. *J Reprod Dev* **55**, 491-495, doi:JST.JSTAGE/jrd/09-069A [pii] (2009).
227 Pal, S. K., Crowell, R., Kiessling, A. A. & Cooper, G. M. Expression of proto-oncogenes in mouse eggs and preimplantation embryos. *Mol Reprod Dev* **35**, 8-15, doi:10.1002/mrd.1080350103 (1993).

228 Voeltz, G. K. & Steitz, J. A. AUUUA sequences direct mRNA deadenylation uncoupled from decay during Xenopus early development. *Mol Cell Biol* **18**, 7537-7545 (1998).
229 Lefresne, J. *et al.* Evidence for multiple sequences and factors involved in c-myc RNA stability during amphibian oogenesis. *Dev Growth Differ* **43**, 195-211, doi:dgd563 [pii] (2001).
230 Janicke, R. U., Sohn, D., Essmann, F. & Schulze-Osthoff, K. The multiple battles fought by anti-apoptotic p21. *Cell Cycle* **6**, 407-413, doi:3855 [pii] (2007).
231 Vousden, K. H. Outcomes of p53 activation--spoilt for choice. *J Cell Sci* **119**, 5015-5020, doi:119/24/5015 [pii]
10.1242/jcs.03293 (2006).
232 Barboza, J. A., Liu, G., Ju, Z., El-Naggar, A. K. & Lozano, G. p21 delays tumor onset by preservation of chromosomal stability. *Proc Natl Acad Sci U S A* **103**, 19842-19847, doi:0606343104 [pii]
10.1073/pnas.0606343104 (2006).