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# Regulation and Function of p53 in Embryonic Stem Cells

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# ABSTRACT

The p53 protein is one of the most well-known tumor suppressor proteins, and it plays a variety of functions in somatic cells. Once activated, p53 induces cell cycle arrest and inhibits cell proliferation. Since it was found that p53 is highly expressed in murine embryonic stem cells, a cell type that proliferates very fast because of a shortened G1 phase, it remained a mystery whether p53 is active in this cell type.

I observed that a significant part of p53 is localized in the nucleus of murine embryonic stem cells and that the majority of this nuclear p53 is bound to DNA. In addition, the anti-proliferative activity of p53 is compromised in stem cells, and this control is due, at least in part, to the high amount of MDMX that is present in embryonic stem cells. This high amount of MDMX is most likely due to exclusion of exon 7 of the *MDMX* RNA during retinoic acid induced differentiation. MDMX co-eluted with p53 from sucrose gradient assays and downregulation of MDMX in mESCs increased MDM2 abundance, a transcriptional target of p53, indicating that MDMX controls p53's transcriptional activity in stem cells. P53 is posttranslationally modified in mESCs and these modifications endow a neutral isoelectric point (pI) of a fraction of the p53 protein that is only present in stem cells. Moreover, according to its nuclear localization in mESCs, p53 influences the transcriptome of mESCs. However, in contrast to the anti-proliferative activity that p53 has in differentiated cells, p53 controls transcription of pro-proliferative genes in embryonic stem cells including *c-myc* and *c-jun*. Chromatin-Immunoprecipitation showed that p53 binds to the responsive element of these proto-oncogenes. The impeded anti-proliferative activity of p53 and the induction of certain proto-oncogenes by p53 in murine embryonic stem cells can explain why stem cells proliferate efficiently despite having high levels of p53.

# ZUSAMMENFASSUNG

Das p53-Protein ist eines der bekanntesten Tumorsuppressorproteine, und es hat eine Vielzahl von Funktionen in somatischen Zellen. Einmal aktiviert, induziert p53 Zellzyklusarrest und Apoptose. In murinen embryonalen Stammzellen (mESCs), einem Zelltyp, der aufgrund einer verkürzten G1 Phase sehr schnell proliferiert, ist p53 sehr stark exprimiert. Es war deshalb bisher unklar, ob p53 in diesen Zelltyp aktiv ist.

Ich beobachtete, dass ein wesentlicher Teil des p53 Proteins im Kern von embryonalen Stammzellen der Maus lokalisiert ist Jedoch ist die anti-proliferative Aktivität von p53 in Stammzellen beeinträchtigt. Dies ist, zumindest teilweise, auf die große Menge an MDMX zurück zu führen, die in embryonalen Stammzellen vorhanden ist. Diese große Menge an MDMX geht wahrscheinlich auf den Ausschluss von Exon 7 der *MDMX* RNA während der Differenzierung zurück. Die Herunterregulierung der MDMX mRNA in mESCs erhöhte die Proteinmenge von MDM2, einem Zielgen von p53. Damit ist es wahrscheinlich, dass MDMX die Transkriptionsaktivität von p53 kontrolliert. Außerdem ist das p53 Protein in mESCs posttranslationell modifiziert. Diese posttranslationellen Modifikationen sorgen für einen neutralen isoelektrischen Punkt (pI) einer Fraktion der p53-Proteine. Im Einklang mit der nuklearen Lokalisation von p53 beobachtete ich, dass p53 das Transkriptom von mESCs beeinflusst. Allerdings beobachtete ich, dass p53 anstelle der anti-proliferativen Aktivität die es in differenzierten Zellen hat, in mESCs die Transkription von pro-proliferative Genen einschließlich c-myc und c-Jun steuert. Chromatin-Immunopräzipitationen zeigten, dass p53 an den Promoter dieser Proto-Onkogene bindet und sich damit wie mutiertes p53 verhält. Die behinderte antiproliferative Aktivität von p53 und die Induktion bestimmter Proto-Onkogene durch p53 in murinen embryonale Stammzellen können erklären, warum sich Stammzellen trotz des hohen p53 Spiegels effektiv vermehren können.

# CONTENT

# 1 INTRODUCTION

Embryonic stem cells are cells that have the capability of differentiating into all kinds of cells. They are particularly present at the beginning of life. Thus any mutation that arises in embryonic stem cells endows a more serious problem since this mutation is carried over into the whole developing organism which is not the case if such a mutation happens in somatic cells. p53, a tumor suppressor protein well-known as "guardian of the genome", plays a critical function in cells, including the maintenance of genomic integrity after genotoxic damage. In embryonic stem cells, p53 is highly abundant but its regulation and function is incompletely understood.

## 1.1 Embryonic stem cells.

Embryonic stem cells (ESCs) are a special type of cells that can be found in all multi-cellular organisms. They can divide and produce more stem cells, a process that is called self-renewal and they can differentiate into diverse specialized cell types. In mammals, two types of stem cells exist, namely embryonic stem cells, which are isolated from the inner cell mass of blastocysts, an early-stage of development and adult stem cells that are found throughout the body after embryonic development. Since adult stem cells are found in matured tissues, they are also called tissue stem cells (Loeffler and Roeder, 2002). Adult stem cells are usually required to replenish dying cells and to regenerate damaged tissues. According to their function, adult stem cells are subdivided into hematopoietic stem cells (Spangrude et al., 1988), mammary stem cells (Liu et al., 2005), intestinal stem cells (van der Flier and Clevers, 2009), mesenchymal stem cells (Phinney and Prockop, 2007), endothelial stem cells (Ferguson et al., 2005), neural stem cells (Stemple and Mahanthappa, 1997) and olfactory stem cells (Murrell et al., 2005).

Principally, the self-renewal capacity and pluripotency are the two main characteristics of stem cells. Self-renewal means that by cell division the stem cells create daughter cells that also have the self-renewal and pluripotency capacity. While ESCs have unlimited self-renewal potential , this is not the case for adult stem cells, which have a more limited self-renewal potential (Thomson et al., 1998; Roobrouck et al., 2008)

Another characteristic of stem cells is their ability to differentiate. While embryonic stem cells differentiate into all derivatives of the three primary germ layers: ectoderm, mesoderm, and endoderm, and into each of the more than 220 cell types in the adult body ( Martin, 1981) possess adult stem cells a limited developmental potential and can only differentiate into cell types of specific lineages (Ulloa-Montoya et al., 2007).

The self-renewal capacity of ESCs is accompanied by rapid proliferation because of a shortened G1 phase (Neganova and Lako, 2008). High levels of CDK2-cyclin A/cyclin E in ESCs allow fast entry of stem cells into S phase. When ESCs start differentiation the G1 phase is extended and cell proliferation is slowed down (Neganova and Lako, 2009).

Research on ESCs became very popular in recent years because of their potential to be used for regenerative medicine. Fifteen years ago, the successful isolation of human ESCs promised to enable the repair of tissue that has been damaged through disease or injury (Donovan and Gearhart, 2001; Thomson and Odorico, 2000; Wobus, 2001). Since that time, scientists predict that directed differentiation of ESCs may facilitate the clinical application of cell transplantation therapy. In January 2009, the US Food and Drug Administration (FDA) approved the first clinical trial for using human ESCs to treat patients with spinal cord injury (Yamanaka, 2009). However, ethical concerns and immunological rejection after allogenic cell transplantation are still main obstacles for the use of ESCs in clinical applications (McLaren, 2001). One way to circumvent these problems is to induce a pluripotent status in somatic cells by

direct reprogramming. By transfection of a combination of transcription factors, cells with stem cell like properties can be made from basically every differentiated cell (Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). These cells are called induced pluripotent stem cells (iPSCs), the third type of stem cells. The iPSCs technology can thus overcome two vital barriers associated with human ESCs: immunological rejection after transplantation and ethical concerns regarding the use of human embryos. Patient-derived iPSCs can generate a variety of somatic cells with the same genetic information as the patient (Jang et al., 2012). These cells can be used to construct disease models and to screen for effective and safe drugs, as well as to treat patients by cell transplantation therapy. Unfortunately, the low frequency of reprogramming and the tendency to induce malignant transformation currently compromises the clinical utility of this powerful approach (Okita et al., 2007).

## 1.2 The tumor suppressor protein p53.

The p53 protein is one of the most well-investigated tumor suppressor proteins. p53 was firstly discovered in 1979 (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). Immediately after its discovery, it was assumed that p53 is a transformation-related protein, which accumulates in the nuclear part of tumor cells and tightly binds to the simian virus 40 (SV40) large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Because of the observed oncogenic activities and its high expression in murine and human tumor cells, *p53* was originally defined as an oncogene (Dippold et al., 1981). However, about ten years after its initial discovery it became clear that the oncogenic p53 that was found in human tumors was actually mutant (Baker et al., 1989; Finlay et al., 1989). Subsequent studies proved that p53 is a tumor suppressor protein that is mutated in over 50% of human carcinomas and to almost 100% in families with the cancer-prone Li-Fraumeni syndrome

(Santibanez-Koref et al., 1991; Sedlacek et al., 1998). As a so-called “cellular gate keeper” and “guardian of the genome”, p53 plays a fundamental role in coordinating the cellular response to a wide range of cellular stresses (Lane, 1992; Levine, 1997).

## 1.3 The domain structure of p53

The p53 protein is composed of 393 amino acids and can be divided into several functional domains including a transactivation domain, a proline-rich domain, a DNA binding domain, a nuclear localization signal, a tetramerization domain, a nuclear export signal and a C-terminal regulatory domain (Boehme and Blattner, 2009). All these domains can be extensively modified with post-translational modifications which modulate p53’s stability and activity (Boehme and Blattner, 2009). In addition, multiple proteins associate with p53. Proteins that interact with the N-terminal transactivation domain of p53 protein are e.g. mouse double minute 2 homolog (MDM2) and mouse double minute 4 homolog (MDMX, also known as MDM4), two negative regulators of p53, p300, an essential histone acetyltransferase, the TATA binding protein (TBP) and the TBP associated factor 9 (TAF9, also known as TAFII31; Chen et al., 1993; Danovi et al., 2004; Finlan and Hupp, 2004; Shi et al., 2009; Jabbur et al., 2002). In proximity to the transactivation domain, (residues 63-97) is the proline-rich-domain, that includes five copies of PXXP, where P represents proline and X any amino acid. This domain is involved in the induction of p53-dependent apoptosis (Sakamuro et al., 1997; Venot et al., 1998; Walker and Levine, 1996; Zhu et al., 1999). and also regulates the stability of the p53 protein (Sakamuro et al., 1997). The DNA binding domain is the main component of the central core of the p53 protein. This domain is required for sequence-specific DNA binding. This domain is furthermore frequently mutated. In fact, over 80% of mutations of the p53 gene are found between the residues 126 and 306 (Cho et al., 1994). The nuclear localization signal (NLS) contains a core of basic amino acids that is surrounded by amino acids

that break the α-helical structure (Kalderon et al., 1984). The NLS is required for the binding to importin-α and allows the translocation of p53 into the nucleus (Gorlich and Mattaj, 1996; Koepp and Silver, 1996; Shaulsky et al., 1990). In proximity to the NLS is the tetramerization domain (TET). This domain is indispensable for p53 binding to DNA and p53's transcriptional activity which can be explained by the fact that p53 monomers bind to DNA in a cooperative manner (Balagurumoorthy et al., 1995). Therefore, the affinity of the p53 protein for its response element is dramatically decreased when the tetramerization domain has been deleted (Balagurumoorthy et al., 1995). Only when p53 is able to form homooligomers, it is able to be fully act as a transcription factor and to activate or suppress distinct target genes that contain p53 sequence-specific binding sites (Davison et al., 1998). The nuclear export signal (NES) is essential for p53 exportation out of the nucleus and its cytoplasmic degradation (Freedman and Levine, 1998). Interestingly, when p53 proteins form tetramers, e.g. in response to DNA damage, the NES signal is blocked (Stommel et al., 1999). The last domain of p53 is the regulatory domain (REG), a domain that has raised the most controversial discussion about its function among the different domains of p53. The first model claims that REG has an allosteric influence on the DBD of the p53 protein (Halazonetis and Kandil, 1993; Hupp and Lane, 1994; Sakaguchi et al., 1997). The second model claims that REG sterically hinders the binding of p53 to unspecific DNA sequences (Anderson et al., 1997). The C-terminal domain of p53 is, extensively posttranslationally modified and these modifications have a regulatory effect on p53 activity (Appella and Anderson, 2000).

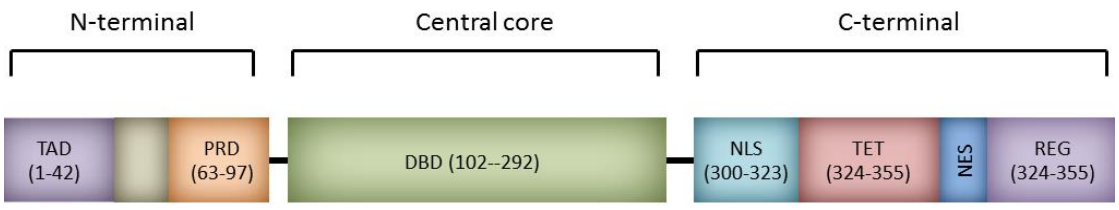


**Figure 1.1 | Schematic structure of the p53 protein.** p53 consists of an N-terminal part, a central core and a C-terminal part. The N-terminal part contains the transactivation domain (TAD), that associates with distinct transcriptional activators and that is required for transactivation activity,

and the proline rich domain (PRD). The DNA binding domain (DBD) builds the core of the p53 protein. The C-terminal tail contains the nuclear localization signal (NLS), the tetramerization domain (TET), the nuclear export signal (NES) and the regulatory domain (REG). Numbers indicate the starting and the terminal amino acid of each domain.

## 1.4 Cellular functions of p53.

As a tumor suppressor protein, p53 primarily prevents inappropriate cell proliferation and maintains genome integrity following genotoxic stress (Vogelstein et al., 2000; Vousden and Lu, 2002). Basically, p53 functions in two ways: one is that p53 is a transcription factor that associates with specific DNA sequences and transactivates a number of target genes whose protein products induce cell cycle arrest, apoptosis and senescence (Riley et al., 2008). In addition to this nuclear activity, p53 also possesses biological activities in the cytoplasm that are transcription-independent. This cytosolic activity of p53 has been identified about a decade of years ago when scientists overexpressed a p53 mutant that lacked most of the DNA-binding domain and that was completely deficient in transactivation function (Haupt et al., 1995). Subsequent studies showed that overexpression of a variety of transactivation-incompetent p53 mutants could indeed efficiently induce apoptosis in human cells (Kakudo et al., 2005). Consistent with these observations, it was found that apoptosis induced by stabilization of an ectopically expressed temperature-sensitive mutant of p53 induced cell death in the absence of RNA and protein synthesis (Caelles et al., 1994). Similarly, p53 was found to trigger apoptosis even in the absence of a nucleus (Chipuk et al., 2003).

### 1.4.1 Transactivation-dependent function of p53.

Based on the phenomena that p53 can provide a transcriptional activation function when fused to a DNA-binding protein GAL4 (Fields and Jang, 1990) and that p53 can bind to DNA (Lane and Gannon, 1983), and such binding was altered in each of 5 human tumor-derived forms of mutant p53 tested (Kern et al., 1991a); p53 was

proposed to have a putative sequence-specific binding function (Kern et al., 1991a). Later, a human DNA sequence was identified to which wild type human p53 specifically binds in vitro (Kern et al., 1991b). Several years ago, forty-eight binding-sites for p53 were observed along chromosome twenty and twenty-two in HCT116 cells that expresses wild type human p53 with high confidentiality (Cawley et al., 2004). By extrapolation, it was predicted that the whole genome may contain approximately 1,600 binding sites for p53 (Cawley et al., 2004). A few years after this estimation, Riley et al. screened for genes that are regulated by p53 and found one hundred and sixty response elements for p53 and one hundred and twenty-nine genes that are regulated by p53 (Riley et al., 2008). Of note, the p53 protein can both activate and repress gene transcription.

#### 1.4.1.1 Transactivation of target genes by p53.

One of the most extensively studied functions of p53 protein is its ability to stimulate RNA polymerase II-dependent gene transcription. This activation of gene transcription by p53 can be explained by several properties of p53. Firstly, p53 is able to bind to DNA (Lane and Gannon, 1983). Secondly, p53 is able to recruit chromatin remodeling factors (Lee et al., 2002) and histone acetyltransferases and methyltransferases to promoters of target genes (An et al., 2004; Avantaggiati et al., 1997; Barlev et al., 2001; Hsu et al., 2004; Lill et al., 1997). These enzymes alter the structure of chromatin by histone modification, and thus allow general transcription factors and the RNA polymerase access to the transcription start site that otherwise would be inaccessible. This model has been supported by showing a physical and functional association of p53 and p300, one of the histone acetyltransferases (Espinosa and Emerson, 2001; Gu and Roeder, 1997; Hsu et al., 2004). The third property is that p53 facilitates the formation of a preinitiation complex by directly binding to the components of the mediator complex (Figure 1.2; Gu et al., 1999). The p53 protein furthermore enhances the affinity of basal transcription factors like TFIIA and TFIID to the promoters of target genes by directly associating with them. This

alters the conformation of the transcription complexes and allows the initiation of transcription (Ko and Prives, 1996; Xing et al., 2001). Most interestingly, the chromatin around p53's response elements is maintained in an open conformation which means that the chromatin is accessible to RNA polymerase both under-stressed and non-stressed conditions (Graunke et al., 1999; Braastad et al., 2003).

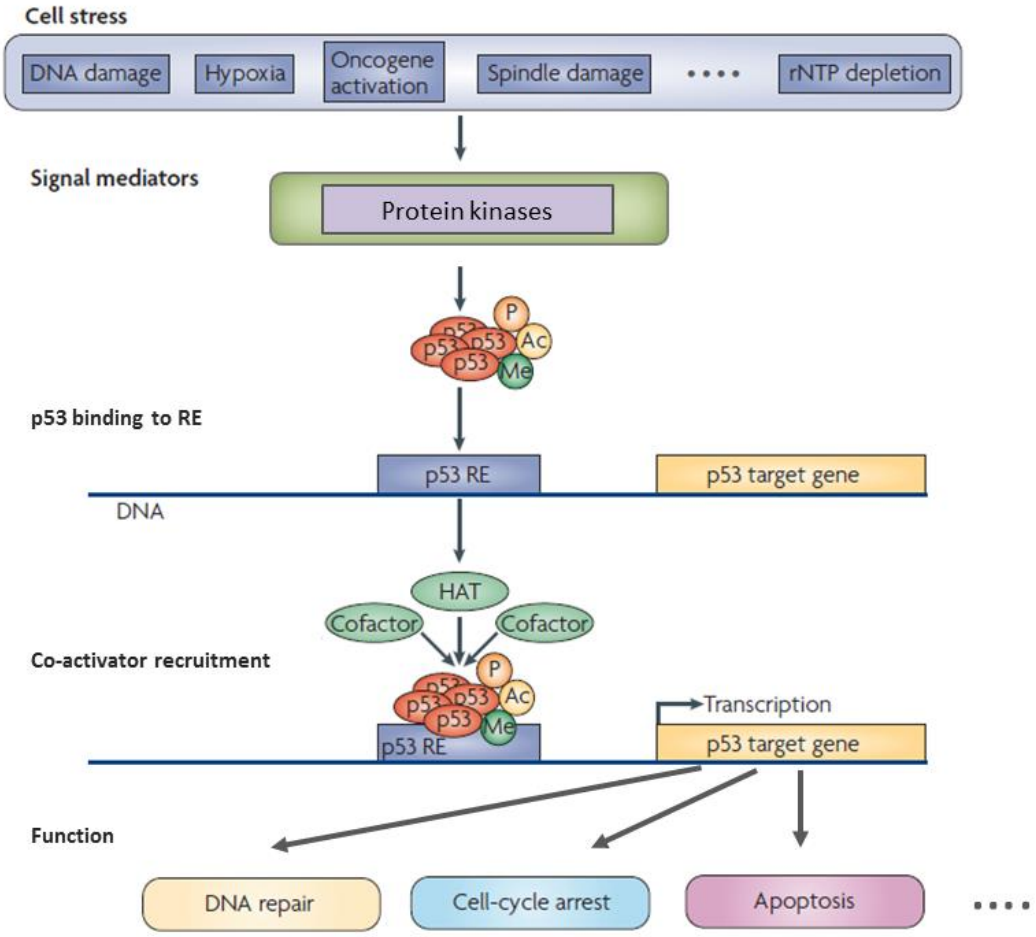


**Figure 1.2 | p53 activation and regulation of target gene expression.** When cells have been exposed to different cellular stresses which have the potential to lead to cell transformation p53 becomes activated. This is in part due to phosphorylation by several kinases at certain residues. Activated p53 then forms tetramers and binds to responsive elements of target genes. The binding of p53 tetramers to responsive elements recruits co-activators, like histone acetyltransferases (HAT) and TATA binding protein-associated factors (TAFs). The most essential pathways that are activated by p53 lead to cell-cycle arrest, senescence and apoptosis (Riley et al., 2008). RE, responsive element; P, phosphorylation; Ac, acetylation; Me, methylation. (Figure from Riley et al., 2008, with modifications)

The p53 protein is activated in response to a variety of cellular signals, including DNA damage, telomere shortening, hypoxia, thermic shock, mitotic spindle damage, unfolded proteins, improper ribosomal biogenesis, nutrition deprivation and even after overexpression of oncogenes (Levine et al., 2006; Vogelstein et al., 2000). In principle, there are three primary outcomes after p53 activation which is cell cycle arrest, apoptosis and senescence respectively. Cell cycle arrest is a transient halt of cell proliferation, which allows the DNA repair machinery to correct mistakes that have occurred during DNA replication or after genotoxic insults prior to the next cell division. Apoptosis leads finally to the killing of the cells whereas senescence interferes with the cells capacity to divide. The choices between these cell fates in a stressed cell depend on a number of variables, which indicates that the p53 pathway may also sense the activity of other signal transduction pathways. In line with this idea induce different cellular stresses distinct groups of p53 target genes (Murray-Zmijewski et al., 2008; Vousden and Prives, 2009). This differential activation of target genes may contribute to the decision about the final fate of the cell after p53 activation.

### 1.4.1.2 Transrepression of target genes by p53.

In addition to its well-studied role in transcriptional activation, p53 has also been shown to repress a wide range of target gene (Burns and El-Deiry, 2003; Mirza et al., 2003; Robinson et al., 2003; Sax et al., 2003; Riley et al., 2008). Currently, four mechanisms are discussed that lead to transcriptional repression by p53. One possibility is that p53 binds directly to the response element of target genes and recruits co-factors that mediate the repression. One of the co-factors that have been shown to mediate transrepression is histone deacetylase 1 (HDAC1). HDAC1 is recruited to promoter regions of target gene via a p53-dependent interaction with mSIN3A (Murphy et al., 1999). The second possibility is that p53 activates transcription of a repressor protein which then inhibits transcription of some genes. For example, p53 regulates p21 expression, which is an inhibitor of cyclin-dependent

kinase (Riley et al., 2008). The induction of p21 inhibits the phosphorylation of the retinoblastoma protein (Niculescu et al., 1998; Xiong et al., 1993) and this inhibition of the phosphorylation of the retinoblastoma proteins keeps E2F-1 in check and thus prevents the transcription of E2F-1 regulated genes (Delavaine and La Thangue, 1999). The third mechanism by which p53 may repress gene transcription is by binding competitively to the response elements and by this hindering transcriptional regulator to access the response elements. For instance, p53 binds to the promoter of the alphafeto protein (AFP), displaces the transcriptional activator and by this represses *afp* expression (Lee et al., 1999). The last possibility for transcriptional repression is by association with other proteins. By this, p53 can associate with the promoter region of some genes which have no p53 responsive element. The cyclin B2 promoter, for instance, contains no p53 response element. However, it contains a NF-Y recognition site. Promoter-bond NF-Y can then interact with p53 and p53 can recruit HDAC1 leading to the repression of the cyclin B2 promoter (Imbriano et al., 2005).

## 1.4.2 Transactivation-independent functions of p53.

In addition to its nuclear functions, p53 also possesses biological activities in the cytosol that are transcription-independent. For instance, p53 translocates to mitochondria in response to hypoxia (Sansome et al., 2001). Here, p53 triggers the assembly of pro-apoptotic factors of the Bcl2 family at the outer membrane of the mitochondria and the formation of multimeric structures resulting in mitochondrial outer membrane permeabilization (MOMP; Leu et al., 2004; Mihara et al., 2003; Moll et al., 2006). MOMP facilitates the release of cytochrome c, a pro-apoptotic protein that is retained in the compartment between the inner and outer membrane of mitochondria (Kroemer et al., 2007; Leu et al., 2004). Principally, apoptosis induced by cytoplasmic p53 does not require p53's transcriptional activity. However, there is clearly a cross-talk or interdependence between the cytoplasmic and nuclear p53 since the sequestration of cytoplasmic p53 by the anti-apoptotic Bcl-$X_L$ protein is regulated

by Puma, which is a target gene of p53 and capable of releasing p53 from the anti-apoptotic Bcl2 proteins to activate Bax (Chipuk et al., 2005).

## 1.5 The regulation of p53 stability and activity.

As p53 is a tumor suppressor protein that inhibits cell proliferation, p53's function has to be tightly controlled to permit normal cell proliferation. Therefore, under normal conditions, p53 abundance is kept at a low level and in an inactive and latent form (Levine, 1997). p53's function can then be induced rapidly through inducing posttranslational modifications (PTMs) and stabilization of the p53 protein.

### 1.5.1 The regulation of p53 stability.

p53's stability is primarily regulated by different ubiquitin-ligases. The most well-studied ubiquitin ligase for p53 is MDM2 which induces both monoubiquitination and polyubiquitination of p53. Polyubiquitinated p53 is then recognized by 26S proteasomes where it is degraded while monoubiquitinated p53 is exported into the cytoplasm (Li et al., 2003; Honda et al., 1997). In addition to MDM2, p53 can also be ubiquitinated by other ubiquitin ligases, such as COP1, PirH2, synoviolin, ARF-BP1, CARP1, CARP2, BAG-2, CHIP and β-TrCP (Chen et al., 2005; Rajendra et al., 2004; Yang et al., 2007, reviewed in Boehme and Blattner, 2009). Apart from its regulation by ubiquitin-modification, p53's stability is also regulated by other small ubiquitin-like proteins, such as SUMO (small ubiquitin-like modifier) (Rodriguez et al., 1999) and NEDD8 (ubiquitin-like modifier) (Xirodimas et al., 2004). Since these modifications are also attached to p53 via lysines, they can inhibit p53 ubiquitination by competing with the same lysine. The stability of p53 is furthermore regulated by phosphorylation, acetylation and methylation (Chehab et al., 2000; Sakaguchi et al., 2000; Schon et al., 2002; Shieh et al., 2000). However, several of these modifications impact on p53 stability only under experimental condition

while their role in a physiological setting is not entirely solved.

The stability of the p53 protein is also controlled by its association with proteins that have no enzymatic activity. These proteins are able to impinge on p53 stability by enhacing or reducing the abundance of enzymes that modify p53 or by altering the affinity between the p53 protein and the modifiying enzymes. Proteins like MIF, G3BP1 and G3BP2 reduce p53 protein degradation and cause its transcriptional inactivation by retaining p53 in the cytoplasm (Jung et al., 2008; Kim et al., 2007). Others like ING1b, S100b and ATF3 disrupt the interaction between p53 and MDM2, and decrease ubiquitinylation of p53, (Fernandez-Fernandez et al., 2005; Leung et al., 2002; Yan et al., 2005). Another notable example is MDMX, a homologe of MDM2 but with no intrinsic ubiquitin ligase activity. MDMX is able to increase or decreease p53's abundance depending on the conditions. MDMX can heterodimerize with the MDM2, and further elevate ubiquitinylation of p53.(Tanimura et al., 1999). But, MDMX can also competitively bind to the N-terminal domain of the p53 protein thus preventing MDM2 mediated ubiquitination and degradation (Barboza et al., 2008; Jackson and Berberich, 2000).

## 1.5.2 The regulation of p53 activity.

p53 is a transcription factor that is recruited to responsive elements of target genes upon cellular stress (McLure and Lee, 1998). Other reports show that p53 occupies the promoter region of target genes regardless of an activating stimulus (Kaeser and Iggo, 2002). If this observation is correct, then p53 may require additional activation steps to be able to trigger gene transcription. One possibility for activating pre-existing p53 molecules at chromatin is to post-translationally modify the protein. Many PTMs including phosphorylation, acetylation and methylation within the N- and C-terminal domain of p53 have been shown to enhance the affinity of p53 for its responsive elements in response to DNA damage, (Chuikov et al., 2004; Gu and Roeder, 1997; Meek, 1999). Phosphorylation of serine-15, for example, which is

implemented in response to double strand breaks, promotes its transcriptional activity by recruiting transcriptional coactivators (Lambert et al., 1998). Mutation of serine 15 therefore reduces the anti-proliferative activity of p53 (Fiscella et al., 1993). Phosphorylation of Serine 392 of p53 is increased in response to UV exposure (Keller and Lu, 2002). This phosphorylation also increases tetramer formation and promotes p53's export into the cytoplasm (Kim et al., 2004; Sakaguchi et al., 1997). Acetylation of p53 at lysine 379 is one of the most important acetylation sites. This modification requires phosphorylation at the N-terminal domain of p53 which then recruits p300/CBP to the C-terminal domain (Lambert et al., 1998). The C-terminal domain of the p53 protein is furthermore methylated by the Set9 methyltransferase. Methylated p53 is restricted to the nucleus and its stability is increased (Chuikov et al., 2004).

Apart from PTMs, p53 activity is also regulated by protein-protein interactions. Notable examples are MDM2 and MDMX, two important negative regulators of the p53 protein (Barak and Oren, 1992; Shvarts et al., 1996). Also several members of the family of tripartite motif proteins (TRIMs) were found to regulate p53's activity. For instance, TRIM13 ubiquitinates MDM2 and leads to its degradation, which leads to the stabilization of p53 and induction of the apoptotic response (Joo et al., 2011). TRIM24 mediates the stabilization and degradation of p53 protein (Allton et al., 2009) and TRIM25 increases p53's stability, while it inhibits p53's transcriptional activity (Zhang et al., 2015).

## 1.6 Similar and distinct functions of MDM2 and MDMX in the regulation of p53.

The mouse double minute 2 homolog protein, MDM2, was first identified as the product of a gene amplified over 50-fold on double minute chromosomes that have been found in a 3T3-DM mouse cell line that has transformed spontaneously

(Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). Later it was found that MDM2 interacts with p53 and regulates p53 activity (Momand et al., 1992). This regulation is performed in two ways: first, MDM2 binds to the transactivation domain of the p53 protein and inhibits p53's transcriptional activity. (Momand et al., 1992); second, MDM2 is an E3 ubiquitin ligase with a RING domain (really interesting new gene) that ubiquitinates p53 (Honda et al., 1997). Ubiquitinated p53 can then be recognized by 26S proteasomes and degraded (Lam et al., 2002), or sequestrated in the cytoplasm (Geyer et al., 2000). Due to the striking p53-inhibiting effect, Mdm2 is regarded as an oncogene. In fact, MDM2 induces tumor formation in nude mice when it is overexpressed (Fakharzadeh et al., 1991). MDM2 and p53 are connected by a negative feedback loop as p53 transactivates *MDM2* and translated MDM2 protein inhibits p53's transcriptional activity (Lozano and Montes de Oca Luna, 1998; Piette et al., 1997). This negative feedback loop is essential for controlling p53's activity and to prevent detrimental pathogenic effects upon excessive p53 activity. The importance of MDM2 and this regulatory feedback loop is demonstrated by the lethality of *MDM2* knockout mice that die because of arbitrary high p53 activity. The lethality of *MDM2* knockout mice is rescued when *p53* and *MDM2* are both knocked-out (Jones et al., 1995; Montes de Oca Luna et al., 1995).

MDMX was identified as a binding partner of p53 by screening a mouse cDNA library (Shvarts et al., 1996). As a close homolog of MDM2, MDMX is another essential negative regulator of p53. Like *MDM2*, *MDMX* acts as an oncogene and induces tumor formation when it is overexpressed (Danovi et al., 2004). MDMX also inhibits p53's transcriptional activity via binding to the transactivation domain of p53 protein. This association of MDMX with p53's transactivation domain also reduces p53 acetylation by p300/CBP, which further inhibits p53's activity (Sabbatini and McCormick, 2002). However, in contrast do MDM2, MDMX has no intrinsic E3 ubiquitin ligase activity and is therefore principally unable to degrade p53.

It is currently unclear how MDM2 and MDMX work together to control p53

abundance and activity. Currently several models exist to explain their cooperation (Figure 1.3). One possibility is that MDM2 and MDMX regulate p53 in a synergistic manner. In this model, the formation of MDM2-MDMX heterodimer is vital for the E3 ubiquitin ligase of Mdm2 (Linares et al., 2003; Linke et al., 2008; Singh et al., 2007). The second possibility is that MDM2 and MDMX regulate p53 in an antagonistic way. MDMX competetively binds to the N-terminal domain of p53 protein, preventing MDM2 mediated ubiquitination and nuclear exportation (Barboza et al., 2008; Jackson and Berberich, 2000). The possibility is that MDM2 and MDMX regulate p53 in an independent manner. Studies have shown that a conditional knockout of either MDM2 or MDMX or both in the central nevous system induces embryonic lehality. However, the timing of the embryonic lethality induced by *MDM2* or *MDMX* knockout (KO) is distinct (Xiong et al., 2006). Furthermore, a conditional KO of *MDM2* in smooth muscle cells caused embryonic lethaliy whereas *MDMX* KO did not show such a severe defect (Boesten et al., 2006).

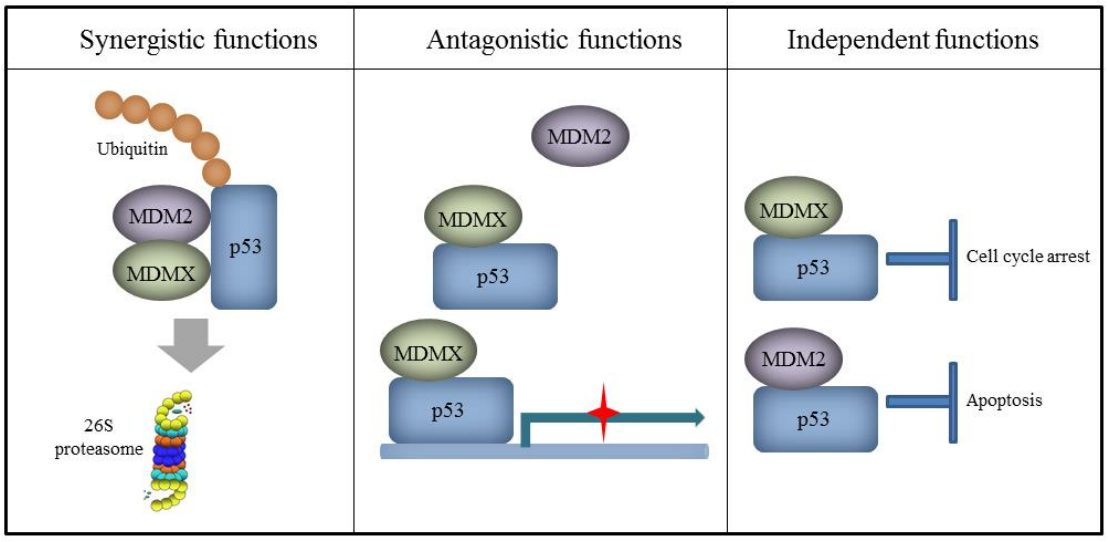


**Figure 1.3 | Models of MDM2 and MDMX function.** In the model of synergistic function, MDM2 and MDMX form a heterodimer to promote p53 degradation. In this model MDM2 and MDMX depend on each other for successful inhibition and ubiquitination of p53. In the model of antagonistic function binds MDMX competitively to the N-terminal domain of p53 and suppresses p53's activity. This prevents MDM2-mediated ubiquitination and degradation of p53. In the model of independent functions play MDM2 and MDMX distinct roles in the regulation of p53.

## 1.7 Mutant p53

The p53 gene is one of the most frequently inactivated genes in human cancers (Hollstein et al., 1991). Approximately 50% of human cancers have a mutation in the *p53* gene leading to inactivation of its function or have lost the *p53* gene completely ( Soussi and Beroud, 2001). About 74% of tumor-derived p53 harbors missense-point mutations that result in a high-level expression of mutant p53 (mutp53), which is usually localized in the nucleus (Olivier et al., 2010). Crystal structure analysis, of some of the p53 mutations has shown that some of these mutants are no longer able to associate with DNA. These mutations are therefore called “contact mutants”. The most commonly changed residues in breast cancer R248Q and R273H belong to this class of p53 mutations. In contrast, R175H and Y220C substitutions generate p53 “structural mutants”. Although the mutation itself is not in the p53/DNA interface, it distorts the structure of the DNA binding domain (DBD) under physiological conditions (Walerych et al., 2012). Careful biophysical studies *in vitro* uncovered a gradient in the extent of p53 DBD destabilization by the specific TP53 hotspot mutations (Bullock et al., 2000). Strikingly, while mutant p53 is no longer able to activate its usual target genes, there is accumulating evidence that mutant p53 acquires novel activities, including distinct DNA-binding and transactivation properties. Indeed, many loci lacking p53-responsive elements are found to be regulated by mutant p53 (Chicas et al., 2000; Scian et al., 2004). For instance, mutant p53 binds to the promoter region of *c-myc* when it is associated with PTEN, CBP and NFY and stimulates the expression of the proto-oncogene (Huang et al., 2013). Mutant p53, moreover, binds preferentially and autonomously to G/C-rich DNA around transcription start sites of several genes, characterized by active chromatin marks (Quante et al., 2012). Binding of mutp53 to these G/C-rich DNA regions that are associated with a large set of cancer-relevant genes may be an initial step in their regulation by mutant p53. In addition, a couple of p53 mutants, although defective in specific DNA sequence binding, retain the capability to bind to non-B DNA structures with high affinity. These DNA structures are rich in repetitive elements and other

sequences with a high likelihood of adopting non-B DNA conformation. Various DNA structures are bound by the different p53 mutants, through distinct mechanisms and with different affinities (Gohler et al., 2005).

In principle, mutant p53 within a cell might have three, not mutually exclusive, outcomes. Firstly, such a mutation will abrogate the tumor suppressor properties of the affected TP53 allele and reduce the capacity of the cell to mount a proper p53 response (Sigal and Rotter, 2000). Secondly, since p53 is active as a tetramer, mutant p53 can have a dominant-negative effect over wild-type p53 by forming mixed tetramers that are incapable of DNA-binding and transactivation. Therefore, even if one WT allele is retained, the cell may be rendered practically devoid of p53 function, particularly since the mutant protein is usually more stable and therefore present in excess over the wild-type counterpart (Michalovitz et al., 1991). Thirdly, mutant p53 possesses activities of its own that are often not present in the original wild-type p53 protein and that can actively contribute to the various aspects of tumor progression (Lanyi et al., 1998; Roemer, 1999). Such an activity is commonly described as mutant p53 gain-of-function (GOF).

An ocean of GOF properties were demonstrated and a variety of underlying mechanisms were proposed after the discovery of the oncogenic potential of mutant p53 (Dittmer et al., 1993; Kim and Deppert, 2004; Shaulsky et al., 1991; Sigal and Rotter, 2000). A pivotal GOF mechanism is the ability of mutant p53 to bind and inactivate the other p53 family members, p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001). These transcription factors have a key role during development in addition to their pro-apoptotic activities (Deyoung and Ellisen, 2007). These p53 family members can at least partially compensate the deletion of p53 because $p53^{+/-}p63^{+/-}$ mice and $p53^{+/-}p73^{+/-}$ mice have reduced survival and show increased metastasis after tumour induction in comparison to $p53^{+/-}$ mice (Flores et al., 2005). Another GOF mechanism is the association of mutant p53 with other transcription factors that bind sequence-specific to target genes and regulate gene transcription. The

association with mutant p53 augments or attenuates the activities of these transcription factors. Well-investigated transcription factors that interact with mutant p53 are SP1 and ETS1 (Kim and Deppert, 2004). Most interestingly, the effects of wild-type and mutant p53 on SP1 and ETS1 are antagonistic (Kim and Deppert, 2004).

## 1.8 p53 isoforms

The p53 protein is encoded by the *TP53* gene, which comprises eleven exons, of which the first one is noncoding, and ten introns. It contains multiple genetic polymorphisms leading to more than one hundred distinct TP53 haplotypes. Some of these haplotypes are correlated with an increased risk of developing cancer (Dumont et al., 2003; Garritano et al., 2010; Wu et al., 2013). The first p53 isoforms were identified in the 1980s (Matlashewski et al., 1984; Wolf et al., 1985). In 1996, an alternatively spliced form of human p53 mRNA containing an additional 133bp exon derived from intron 9 was discovered. This splice variant encodes a truncated protein of 341 amino-acids that contains ten new amino-acids that are derived from the novel exon. The truncated protein, which lacks part of the p53 tetramerization domain, fails to bind DNA in vitro (Flaman et al., 1996). Today twelve isoforms are known that are encoded by the *p53* gene (Bourdon et al., 2005; Courtois et al., 2002; Flaman et al., 1996; Yin et al., 2002).

As I described in Section 1.3, p53, comprises 393 amino acids and this isoform is named p53, FLp53, p53α or TAp53α; Figure 1.4). Other p53 isoforms are the result of alterative splicing, alternative promoter usage and alternative initiation of translation (Marcel et al., 2011). The proximal promoter (P1) controls the expression of the p53 isoforms p53α, p53β and p53γ) as well as the isoforms that lack the first 40 amino acids (Δ40p53α, Δ40p53β, Δ40p53γ; Figure 1.4). The internal promoter (P2) controls the expression of the p53 isoforms that lack the first 133 amino acids (Δ133p53α,

Δ133p53β, Δ133p53γ) and the isoforms that lack the first 160 amino acid (Δ160p53α, Δ160p53β, Δ160p53γ) (Figure 1.4). The complete exclusion of intron 9 generates the canonical p53 isoform (α isoforms) while partial retention of intron 9 endows the -β and –γ isoforms. The β isoforms entirely replaced the tetramerisation domain and the regulatory domain by fifteen new amino acids (Marcel et al., 2011).

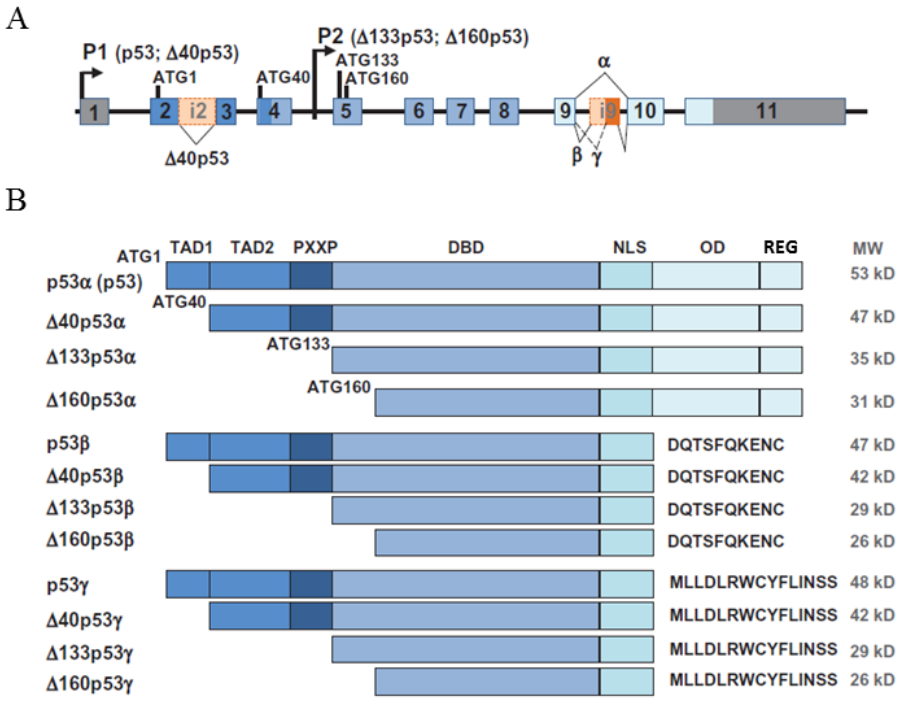


**Figure 1.4 | p53 isoforms encoded by the human *p53* gene. A.** Schematic drawing of the human p53 gene. The human p53 gene is composed of eleven exons. Transcription driven by two different promoters (P1 and P2) and alternative splicing at splicing sites i2 and i9 give rise to four different translation initiation sites (ATG1, ATG40, ATG133, ATG160). **B.** Domain structure of the different p53 isoforms. TAD1: transactivation domain 1 (amino acid 1-42), TAD2: transactivation domain 2 (amino acid 43-63), PXXP: proline rich domain (amino acid 64-92), DBD: DNA binding domain (amino acid 102-306), NLS: nuclear localization signal (amino acid 316-325), OD: oligomerization domain (amino acid 307-355) REG: regulatory domain (amino acid 364-393). MW: molecular weight; kD: kilo Dalton. (Surget et al., 2013, with modifications).

The tetramerization domain and the C-terminal regulatory domain of p53 are important for the regulation of the subcellular localization of p53, which affects p53 activity. In addition, this area contains ubquitination sites for most of those E3 ligases that modify p53 and is thus important for p53 stability (Boehme and Blattner, 2009). The absence of the tetramerization and regulatory domains furthermore alters the

choice for target genes. p53β, for instance, which lacks the last sixty amino acids of full length p53 while ten other amino acids have been added binds preferentially to the *bax* promoter but binds poorly to the *MDM2* promoter (Bourdon et al., 2005). Likewise, p53γ that also lacks the last sixty amino acids of full length p53 while it has fifteen new amino acids, strongly binds to the *bax* promoter, but not to the *p21* promoter (Bourdon et al., 2005). p53 isoforms can furthermore affect the activity of full length p53. The Δ40p53α isoform, for example, which is generated by alternative splicing of intron 2, and lacks the first forty amino acids and thus most of the transactivation domain, has a dominant-negative effect over p53 and inhibits its transcriptional activity and impairs p53-mediated growth suppression (Courtois et al., 2002). Δ40p53α furthermore affects ubiquitination and subcellular localization of full length p53 (Ghosh et al., 2004). Interestingly, mice heterozygous for the Δ40 isoform (p53/Δ40p53) are less susceptible to cancer than heterozygote $p53^{+/-}$ mice. However, mice homozygous for the Δ40 isoform (Δ40p53/Δ40p53) are as cancer prone as $p53^{-/-}$ mice and do not show any accelerated aging, indicating that the accelerated aging phenotype may depend on the interplay between Δ40p53 and wild-type p53 (Maier et al., 2004; Tyner et al., 2002).

While the human isoforms of p53 are quite well investigated, less is known about these isoforms in mice. The use of the two promoters P1 and P2 yet appears to be conserved as well as the alternative splicing leading to the Δ40 and Δ160 isoforms (Δ157 in mice; Marcel et al., 2011). Yet the absence of the oligomerization domain and the regulatory domain, giving rise to the γ isoform that has not been found as yet in mouse cells and also the Δ133 isoform has not been described as yet. Instead, in mouse fibroblasts, another p53 isoform has been found that is generated by alternative splicing using a cryptic 3' splicing site of exon eleven, giving rise to the p53 AS isoform (Figure 1.5, Wolf et al., 1985). Several papers reported that p53 and p53AS bind both to the responsive element of p53 but have distinct biochemical activities and are functional different. For instance, in contrast to p53 which binds poorly to DNA in vitro, the p53AS protein has a much higher affinity for DNA (Miner and

Kulesz-Martin, 1997; Wolkowicz et al., 1995; Wu et al., 1994).

The different isoforms of murine p53 can be detected by an isoform-specific antibody (SAPU) that recognizes to the N-terminal and C-terminal domain of p53 (Marcel et al., 2013).

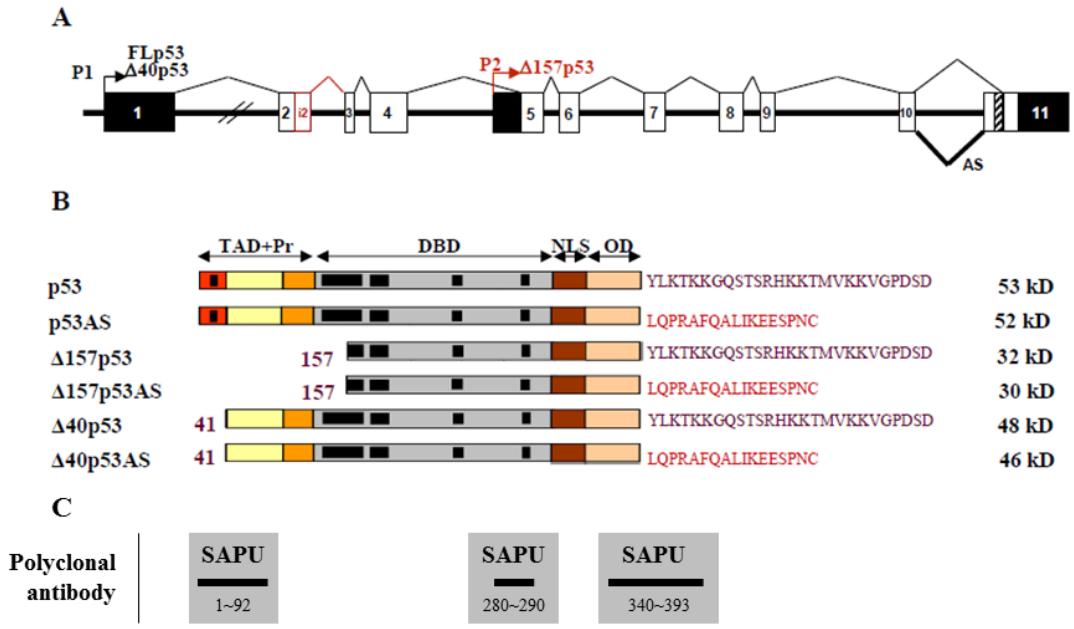


**Figure 1.5 | p53 isoforms encoded by the mouse *p53* gene. A.** Schematic drawing of the p53 gene showing the 11 exons and 10 introns and the two active promoters (P1) and (P2). The alternative splicing of intron 2 (i2) and the internal promoter (P2) are marked in red. Alternative splicing of the C-terminus generates the AS isoforms where the C terminal domain is replaced with 17 new amino acids (p53AS, Δ157p53AS, Δ40p53AS). **B.** Schematic drawing of the different isoforms. p53 and p53AS genes are transcribed from promoter P1 and contain the transactivation domain and the proline-rich domain. Δ157p53 andΔ157p53AS are truncated isoforms that are transcribed from the internal promoter P2 and that lack the transactivation domain as their translation is initiated at ATG-157. Δ40p53 and Δ40p53AS are transcribed from promoter P1, yet truncated due to alternative initiation of translation. Translation is initiated at ATG-41 and the proteins still contain part of the transactivation domain. The amino acid sequence in the C-terminal end is written in red and purple (AS). Molecular weights (kD) of the different p53 isoforms are indicated. TAD+Pr: transactivation domain and proline rich domain; DBD: DNA binding domain; NLS: nuclear localization domain; OD: oligomerization domain. **C.** Binding sites of the isoformspecific anti-p53 antibody SAPU. (Marcel et al., 2013; with modifications).

## 1.9 p53 in stem cells.

Embryonic stem cells (ESCs), the cell type derived from the inner cell mass of a blastocyst, hold the potential that differentiate into all three germ layer of an embryo (Evans and Kaufman, 1981; Martin, 1981). Owing to the ability of differentiating into all kinds of cell types, ESCs have developed mechanisms to adapt to various cellular stress, in particular DNA damage insults, to avoid passing the mutation to their progeny cells (Cervantes et al., 2002). As a guardian of the genome, p53 plays a crucial role in maintaining the integrity of genetic information (Lane, 1992). In embryonic stem cells, p53 exerts an important function in maintaining genomic stability and regulating differentiation, DNA repair and apoptosis (Li and Huang, 2010; Lin et al., 2005). Like differentiated cells, stem cells activate a p53-dependent stress response (Solozobova et al., 2009). Thus, inhibition of p53 activity by treatment of cells with pifithrin-α, a small molecule inhibitor of p53, or shifting a temperature-sensitive mutant of p53 to the non-permissive temperature, reduces apoptosis in mESCs (Lee et al., 2005; Sabapathy et al., 1997). However, while ESCs are capable of inducing apoptosis in response to DNA damage, they lack the p53-dependent G1/S checkpoint in response to DNA damage (Aladjem et al., 1998). In comparison to differentiated cells, ESCs have two unique properties regarding their response to DNA damage that are regulated by p53. In the first place, ESCs are more sensitive to DNA damaging agents than somatic cells (Cervantes et al., 2002). Secondly, p53-dependent repression of Nanog and Oct3/4 leads to the differentiation of mESCs that have been exposed to DNA damage (Lin et al., 2005; Qin et al., 2007).

Apart from the notion that p53 maintains the genome integrity of ESCs, a number of studies substantiate the finding that p53 is highly expressed in ESCs (Sabapathy et al., 1997; Solozobova and Blattner, 2010).

In addition to ESCs, p53 also plays a key role in adult stem cells (ASCs), For example, p53 regulates epithelial-mesenchymal-transition (EMT) in the mammary gland. EMT

and the reversed processes mesenchymal-epithelial-transition (MET) are key process for the regulation of embryogenesis. p53 suppresses EMT by binding to the promoter region of the microRNA miR-200c and elevating its transcription level (Chang et al., 2011).

Besides the regulation of differentiation and apoptosis in ESCs, p53 also serves as a barrier for the generation of iPSCs. A series of reports demonstate that inhibition of p53-regulated apoptosis pathway increase the efficiency of iPSC generation significantly (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009). However, the process of reprogramming by inhibition of p53 expression remains obscure because p53 will be required to eliminate damaged and unhealthy iPS cells and their progeny.

## 1.10 Aim of this project.

Despite the high abundance of the anti-proliferative p53 protein, mESCs have a high proliferation rate. A potential reason for this caveat is that p53 shows a predominantly cytoplasmic localization in stem cells while it is mostly nuclear in differentiated cells. The primary aim of this project was therefore:

(1) To elucidate whether p53 indeed has a cytoplasmic localization in stem cells or whether this is just a question of the ratio of cytoplasm and nucleus, which is different in stem cell, and differentiated cells.

(2) To find out how stem cells can survive despite expressing such a high amount of p53. E.g. to see whether p53 is differently modified in mESCs and/or differently associated with other protein,

(3) To investigate the ultimate function of p53 in mESCs.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals

| Chemical | Source |
|---|---|
| Acetic acid | Merck, Darmstadt |
| Acetone | Roth, Karlsruhe |
| Ammonium persulfate (APS) | Sigma-Aldrich, Taufkirchen |
| Agar | Nordwald, Erlangen |
| Agarose | PeqLab, Erlangen |
| Ampicillin, sodium salt | Roth, Karlsruhe |
| Aproptinin | Sigma-Aldrich, Taufkirchen |
| β-mercaptoethanol | Roth, Karlsruhe |
| β-mercaptoethanol for cell culture | Invitrogen, Karlsruhe |
| Bovine serum albumin (BSA) | PAA, Cölbe |
| Bromophenol blue | Sigma-Aldrich, Taufkirchen |
| Deoxyribonucleic acid sodium salt from salmon testes | Sigma-Aldrich, Taufkirchen |
| Dimethylsulfoxide (DMSO) | Roth, Karlsruhe |
| 6×DNA loading dye | PeqLab, Erlangen |
| dNTPs | Roche, Mannheim |
| Draq5 | BioStatus Limited, Shepshed (UK) |
| Dithiothreitol (DTT) | Sigma-Aldrich, Taufkirchen |
| Dulbecco's Modified Eagle Medium (DMEM) | Invitrogen, Karlsruhe |
| GlutaMAX Dulbecco's Modified Eagle Medium (GlutaMAX DMEM) | Invitrogen, Karlsruhe |
| Formaldehyde | Merck, Darmstadt |

| | |
|---|---|
| EDTA 25 mM | Fermentas, St. Leon-Roth |
| Ethylenediamine tetra-acetic acid (EDTA) | Roth, Karlsruhe |
| Ethanol | Roth, Karlsruhe |
| Etoposide (Eto) | Sigma-Aldrich, Taufkirchen |
| Fast Red TR Salt | Sigma-Aldrich, Taufkirchen |
| Fetal Bovine Serum (FBS) | PAA, Cölbe |
| Fetal Bovine Serum for mES cells (FBS) | PAN-Biotech GmbH, Aidenbach |
| Glycerol | Roth, Karlsruhe |
| Glycine | Roth, Karlsruhe |
| HEPES | Roth, Karlsruhe |
| HYDROMOUNT™ | National diagnostics, Hessisch Oldendorf |
| ImmunoPureR Immobilized Protein A | Pierce, Thermo Scientific, Rockford (USA) |
| Isopropanol | Roth, Karlsruhe |
| Leptomycin B (LMB) | Sigma-Aldrich, Taufkirchen |
| Leupeptin | Sigma-Aldrich, Taufkirchen |
| Lithium chloride (LiCl) | |
| MACSfectin | Miltenyi Biotec, Bergisch Gladbach |
| Magnesium carbonate ($MgCO_3$) | Roth, Karlsruhe |
| Magnesium chloride ($MgCl_2$) | Roth, Karlsruhe |
| Methanol | Roth, Karlsruhe |
| N,N,N',N'-Tetramethylethylenediamine (TEMED) | Roth, Karlsruhe |
| Naphthol AS-MX phosphate | Sigma-Aldrich, Taufkirchen |
| N-Ethylmaleimide (NEM) | Sigma-Aldrich, Taufkirchen |
| Nicotinamide (NA) | Sigma-Aldrich, Taufkirchen |
| Non-Essential Amino Acid (NEAA) | Invitrogen, Karlsruhe |
| Nonidet-P40 (NP-40) | Roth, Karlsruhe |
| NuPAGE® Antioxidant | Invitrogen, Karlsruhe |

| | |
|---|---|
| NuPAGE® LDS Sample Buffer | Invitrogen, Karlsruhe |
| NuPAGE® MOPS SDS Running buffer | Invitrogen, Karlsruhe |
| Nutlin-3a | Sigma-Aldrich, Taufkirchen |
| Penicillin/Streptomycin | Invitrogen, Karlsruhe |
| 1,10-Phenanthtroline | Sigma-Aldrich, Taufkirchen |
| Phenylmethanesulfonyl fluoride (PMSF) | Sigma Aldrich, Taufkirchen |
| Phosphostop Phosphatase Inhibitor Cocktail Tablets | Roche, Grenzach |
| Phosphate buffered saline (PBS) | Invitrogen, Karlsruhe |
| Potassium chloride (KCl) | Roth, Karlsruhe |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Roth, Karlsruhe |
| Rotiphorese Gel 30: Acrylmide/bis-acrylamide | Roth, Karlsruhe |
| Roti®-Mark BI-Pink | Roth, Karlsruhe |
| Roti®-Quant (5×) | Roth, Karlsruhe |
| rRNasin RNase Inhibitor | Promega, Mannheim |
| Skimmed milk powder | Saliter, Oberg ünzburg |
| Sodium acetate (NaAc) | Roth, Karlsruhe |
| Sodium bicarbonate ($NaHCO_3$) | Roth, Karlsruhe |
| Sodium chloride (NaCl) | Roth, Karlsruhe |
| Sodium dodecyl sulfate (SDS) | Roth, Karlsruhe |
| Sodium hydrogen phosphate ($Na_2HPO_4$) | Roth, Karlsruhe |
| Sucrose | Roth, Karlsruhe |
| Thio-urea | Roth, Karlsruhe |
| Trichloroacetic acid (TCA) | Sigma-Aldrich, Taufkirchen |
| Trichostatin A (TSA) | Sigma-Aldrich, Taufkirchen |
| Tris base | Roth, Karlsruhe |
| Triton X-100 | Roth, Karlsruhe |
| TRizol | Invitrogen, Karlsruhe |
| tRNA | Sigma-Aldrich, Taufkirchen |

| | |
|---|---|
| Tryptone/Peptone | Roth, Karlsruhe |
| Tween 20 | Roth, Karlsruhe |
| Urea | Roth, Karlsruhe |

## 2.1.2 Enzymes

| Enzyme | Source |
|---|---|
| DNase I | Pierce, Karlsruhe |
| M-MLV Reverse Transcriptase, RNase H(-) | Promega, Mannheim |
| RNase A | Invitrogen, Karlsruhe |
| RQ1 DNase I | Promega, Mannheim |
| 0.25% Trypsin-EDTA | Invitrogen, Karlsruhe |
| GoTaq® G2 DNA Polymerase | Promega, Mannheim |

## 2.1.3 Kits

| Kits | Source |
|---|---|
| RNeasy Mini Kit (RNA purification) | Qiagen, Hilden |
| Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit | Bio-Rad, München |

## 2.1.4 Oligonucleotides

### 2.1.4.1 Primers for qRT-PCR

All primers were synthesized by Metabion (Martinsried).

| Name | Sequence |
|---|---|
| RT-akt1-forward | 5'-TGC ATT GCC GAG TCC AGA A-3' |
| RT-akt1-reverse | 5'-CAG CGC ATC CGA GAA ACA-3' |
| RT-igf2-forward | 5’-CGT GGC ATC GTG GAA GAG T-3’ |
| RT-igf2-reverse | 5’-ACA CGT CCC TCT CGG ACT TG-3’ |
| RT-c-jun-forward | 5’-CGA GTA CTG AAG CCA AGG GTA CAC-3’ |
| RT-c-jun-reverse | 5’-TGA GAT CGA ATG TTA GGT CCA TGC-3’ |
| RT-lef1-forward | 5’-CCC ACA CGG ACA GTG ACC TA-3’ |
| RT-lef1-reverse | 5’-TGG GCT CCT GCT CCT TTC T-3’ |
| RT-mdm2-forward | 5’-TGG AGT CCC GAG TTT CTC TG-3’ |
| RT-mdm2-reverse | 5’-AGC CAC TAA ATT TCT GTA GAT CAT TG-3’ |
| RT-c-myc-forward | 5’-GTC GTA ATT CCA GCG AGA GAC A-3’ |
| RT-c-myc-reverse | 5’-CTC TGC ACA CAC GGC TCT TC-3’ |
| RT-p21-forward | 5’-CCT GAC AGA TTT CTA TCA CTC CA-3’ |
| RT-p21-reverse | 5’-CAG GCA GCG TAT ATC AGG AG-3’ |
| RT-ribpo-forward | 5’-GGA CCC GAG AAG ACC TCC T-3’ |
| RT-ribpo-reverse | 5’-GCA CAT CAC TCA GAA TTT CAA TGG-3’ |

### 2.1.4.2 Primers for chromatin-immunoprecipitation assay

All primers were synthesized by Metabion (Martinsried).

| Name | Sequence |
|---|---|
| ChIP-akt1-forward | 5’-CCA AGC CTC ACC CAT CTG A-3’ |
| ChIP-akt1-reverse | 5’-GCG TGG GAA GTG AAT CAG TTT-3’ |
| ChIP-c-jun-forward | 5’-TCC GAC AGA CTC CGC AAG-3’ |
| ChIP-c-jun-reverse | 5’-TGA GTC CTT ATC CGA CCT GAG-3’ |
| ChIP-mdm2-forward | 5’-CGA GAG GTG ACA GGT GCC-3’ |
| ChIP-mdm2-reverse | 5’-CAG GAC TTA GCT CCT CCG AC-3’ |

| | |
|---|---|
| ChIP-c-myc-forward | 5'-GTA AGC ACA GAT CTG GTG G-3' |
| ChIP-c-myc-reverse | 5'-TGG TAA GTC AGA AGC TAC GGA-3' |

### 2.1.4.3 siRNA

All siRNAs were synthesized by Eurofin MWG Operon (Ebersberg).

| Name | Sequence |
|---|---|
| MDM2 siRNA | 5'- GGC AAA AAG CUG ACA GAG A -3' |
| MDMX siRNA | 5'- AGA TTC AGT TGG TTA TTA A -3' |
| p53 siRNA | 5'-GCA UGA ACC GGA GGC CCA U-3' |
| Control siRNA | 5'-AAC CCC UUU UAA AAG GGG CCC-3' |

## 2.1.5 Eukaryotic cells and cell lines

| Name | Source and description |
|---|---|
| mESCs | Embryonic stem cells from a mouse of the D3 strain |
| Diff. cells | mESCs induced to differentiate by incubation with retinoic acid for 7 days |
| Feeder cells | Mouse embryonic fibroblasts that have been irradiated with 6.3 Gray of ionizing radiation |
| MEF *p53*$^{+/+}$ | Mouse embryonic fibroblasts |
| MEF *p53*$^{-/-}$ | Embryonic fibroblasts from a mouse with a homozygous deletion of the *p53* gene |
| *p53*$^{-/-}$ mESCs | Embryonic stem cells from a mouse of the D3 strain with a homozygous deletion of the *p53* gene |

## 2.1.6 Antibodies

### 2.1.6.1 Primary antibodies

| Primary antibodies | Description | Source |
|---|---|---|
| α7 (MCP72) | Mouse, monoclonal | Enzo Life Sciences GmbH, Lörrach |
| Acetyl-p53 (Lys379) | Rabbit, polyclonal | Cell Signaling, Danvers (USA) |
| β-actin | Mouse, monoclonal | Abcam, Cambridge (UK) |
| β-actin (I-19) | Goat, polyclonal | Santa Cruz, Heidelberg |
| Bax | Rabbit, polyclonal | Cell Signaling, Danvers (USA) |
| GAPDH (6C5) | Mouse, monoclonal | HyTest, Köln |
| Histone H3 (C16) | Goat, polyclonal | Santa Cruz, Heidelberg |
| c-Jun (H79) | Rabbit, polyclonal | Santa Cruz, Heidelberg |
| MDM2 (4B2) | Mouse, monoclonal | Calbiochem, Merck, Darmstadt |
| MDMX (82) | Mouse, monoclonal | Sigma-Aldrich, Taufkirchen |
| Nanog (C4) | Mouse, monoclonal | Santa Cruz, Heidelberg |
| Oct3/4 (C10) | Mouse, monoclonal | Santa Cruz, Heidelberg |
| p53 (1C12) | Mouse, monoclonal | Cell Signaling, Danvers (USA) |
| p53 (PAb246) | Mouse, monoclonal | Millipore, Schwalbach |
| p53 (PAb421) | Mouse, monoclonal | Millipore, Schwalbach |
| p53 (CM5) | Rabbit, polyclonal | Vector-Lab., Peterborough (UK) |
| PARC (PO69) | Mouse, monoclonal | BioLegend, Fell |
| PCNA (PC10) | Mouse, monoclonal | Santa Cruz, Heidelberg |
| Phospho-p53 (Ser6) | Rabbit, polyclonal | Cell Signaling, Danvers (USA) |
| Phospho-p53 (Ser15) | Rabbit, polyclonal | Cell Signaling, Danvers (USA) |
| Phospho-p53 (Ser392) | Rabbit, polyclonal | Cell Signaling, Danvers (USA) |

### 2.1.6.2 Secondary antibodies

| Secondary antibodies | Source |
|---|---|
| Goat anti mouse IgG/HRP | Dako, Glostrup (Dennmark) |
| Goat anti rabbit IgG/HRP | Dako, Glostrup (Dennmark) |
| Sheep anti goat IgG/HRP | Dako, Glostrup (Dennmark) |
| Alexa Fluor 488 goat anti-mouse IgG (H+L) | Invitrogen, Karlsruhe |
| Alexa Fluor 488 goat anti-rabbit IgG (H+L) | Invitrogen, Karlsruhe |

## 2.1.7 Size standards

| Name | Source |
|---|---|
| PeqGOLD Protein Marker IV | PeqLab, Erlangen |
| PeqGOLD 100bp DNA-Ladder Plus | PeqLab, Erlangen |

## 2.1.8 Instruments and consumables

| Name | Source |
|---|---|
| Amersham ECL Hyperfilm | GE Healthcare, Freiberg |
| Analytical balance | Mettler Toledo, Gießen |
| Bioruptor® sonication device | Diagenode, Liège (Belgium) |
| Blotting Filter Paper | Bio-Rad, München |
| Cell culture incubator | Heraeus, Fellbach |
| Cell culture plastic ware (flask and dishes) | Greiner Bio-One, Frickenhausen |
| Clean bench | W. H. Mahl, Trendelburg |
| Cold room (4°C) | Foster, Schutterwald |

| | |
|---|---|
| Cold room (-20°C) | Foster, Schutterwald |
| Cooling centrifuge Biofuge PrimoR for 15/50 ml falcons | Heraeus, Fellbach |
| Cooling centrifuge Eppendorf 5417 R for PCR tubes | Eppendorf, Hamburg |
| Cooling microcentrifuge Heraeus Fresco17 | Thermo Scientific, Waltham (USA) |
| Corex® Centrifuge Tubes | Corex (USA) |
| Covaris™ S220 focused ultrasonicator | Thermo Scientific, Waltham (USA) |
| Electrophoresis equipment (SDS-PAGE) | Bio-Rad, München |
| Electrophoresis Power Supply | PeqLab, Erlangen |
| ELx 808IU Ultra Microplate Reader | Bio-Tek Instruments, Bad Friedrichshall |
| End-over-end rotator | Heidolph, Schwabach |
| Eppendorf tubes (1.5 ml and 2 ml) | Eppendorf, Hamburg |
| Ettan IPGphor II Isoelectric Focusing system | GE Healthcare, Freiberg |
| Falcons (15 ml and 50 ml) | Greiner Bio-One, Frickenhausen |
| Floor centrifuge Beckman Avanti J-20 | Beckman Coulter, Krefeld |
| Freezer (-20 °C) | Liebherr, Ochsenhausen |
| Freezer (-80 °C) | New Brunswick Scientific, Edison (USA) |
| Glass pipettes | Brand, Wertheim |
| Glassware (Erlenmeyer flasks, beakers, bottles) | Scott, Mainz |
| Hamilton syringe | Hamilton, Martinsried |
| High speed floor centrifuge Avanti J2-HS | Beckman Coulter, Krefeld |
| Horizontal nucleic acid electrophoresis system | Bio-Rad, München |
| Immun-Blot® PVDF membrane | Bio-Rad, München |
| Ink | Pelikan, Hannover |
| Inverted microscope | Leica, Wetzlar |

| | |
|---|---|
| KODAK X-OMAT 2000 X-ray film processor | Kodak, Stuttgart |
| Laboratory balance | Sartorius, Göttingen |
| Magnetic stirrer | IKA Labortechnik, Stauffen |
| Measuring cylinders (10 ml, 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1000 ml, 2000 ml) | Brand, Wertheim |
| Microcentrifuge Heraeus Pico 21 | Thermo Scientific, Waltham (USA) |
| NanoDrop ND-1000 Spectrophotometer | Marienfeld, Lauda-Königshofen |
| Neubauer counting chamber | Marienfeld, Lauda-Königshofen |
| NuPAGE® 10% Bis-Tris polyacrylamide gel | Invitrogen, Karlsruhe |
| Parafilm M | Brand, Wertheim |
| PCR thermocycler | Bio-Rad, München |
| 96-well PCR plate and q-PCR clear seal | Steinbrenner, Wiesenbach |
| Petri dishes | Greiner Bio-One, Frickenhausen |
| Pipettes (2-20µl, 20-200µl, 200-1000µl) | Gilson, Middleton (USA) |
| Pipette Aid | Brand, Wertheim |
| Pipette tips | Brand, Wertheim |
| Platform shaker | Heidolph, Schwabach |
| Real-time PCR System StepOnePlus | Applied Biosystems, Darmstadt |
| Refrigerator (4 °C) | Liebherr, Ochsenhausen |
| Shaker for Eppendorf tubes | Eppendorf, Hamburg |
| Syringes and needles | B. Braun, Melsungen |
| Test-tube rotator | Kisker, Steinfurt |
| Thermomixer | Eppendorf, Hamburg |
| Trans-Blot® Turbo™ Transfer System | Bio-Rad, München |
| UV transilluminator | PeqLab, Erlangen |
| Vortexer | Julabo, Seelbach |

### 2.1.9 Software

| Name | Version/Description |
|---|---|
| Bowtie | V 0.12.7 |
| CASAVA | V 1.8.1 |
| Filezilla | V 3.9.0.2 |
| HTSeq | V 0.5.3p3 |
| ImageJ | V 1.45s |
| Primer Primier | V 5.0-64bit |
| R | DESeq |
| TOPHAT | V 1.4.1 |
| Zeiss LSM Image Browser | V 4.2.0.121 |

### 2.1.10 Data Base

| Name | Source |
|---|---|
| Ensembl Release 67 | Ensembl |
| Mouse genome M37 | NCBI |

# 2.2 Methods

## 2.2.1 Cell Culture

### 2.2.1.1 Cultivation of mouse embryonic fibroblasts

The *p53*$^{+/+}$ and *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (PAA) and 1% penicillin/streptomycin (Invitrogen) at 37$^{o}$C in 5% $CO_2$ and 90% humidity. When the cells reached 80-90% confluence, they were trypsinized and subcultured in a new cell culture flask. For trypsinization, the cell culture medium was aspirated, and the cells were washed once with PBS (Invitrogen). Then the cells were incubated with trypsin-EDTA (Invitrogen) at 37$^{o}$C until they were detached from the cell culture dish. The trypsinization was stopped by addition of five volumes of complete growth medium. The cells were pelleted at 1,200 rpm for 2 minutes. The supernatant was aspirated and the cell pellet was suspended in complete cell culture medium. A desired number of MEFs were transferred into a new cell culture flask.

### 2.2.1.2 Preparation of feeder cells

#### 2.2.1.2.1 Isolation of primary embryonic fibroblasts

Prior to the isolation of mouse embryonic fibroblasts, the surgical instruments were autoclaved. During the isolation process, the instruments were kept in 70% ethanol.

A pregnant mouse was sacrificed 13.5 days postcoitum by cervical dislocation (done by Selma Huber). The body of the mouse was opened and the uterine horns were dissected and transferred into a sterile petri dish containing sterile PBS. The embryos were peeled from the placenta and surrounding tissue with forceps and each embryo was placed into a new sterile petri dish containing PBS. The embryo was separated from yolk and the head, tail and liver were removed. The rest of the embryo was

transferred into a 15 ml-reaction tube and chopped with a blade until no large pieces of tissue were visible anymore. The chopped embryos were incubated with 2 ml 0.25% Trypsin-EDTA at 37°C for 10-15 minutes. The trypsin was inactivated by addition of 4 ml complete cell culture medium and the larger pieces were separated from single cells and small cell clumps by sedimentation for 5 minutes. The supernatant containing single cells was carefully aspirated and transferred into a new 15 ml-reaction tube. Then the cells were collected by centrifugation at 1,200 rpm for 5 minutes. The cell pellet was suspended in 10 ml of cell culture medium and transferred into a 10 cm cell culture petri dish. The cells were cultured at 37°C and 5% $CO_2$ in a humidified cell culture incubator.

#### 2.2.1.2.2 Amplification of primary fibroblasts

When the fibroblasts reached 80-90% confluency, the cells were passaged at a ratio of 1:6 (P1 cells) and incubated at 37°C with 5% $CO_2$ and 90% humidity or frozen in liquid nitrogen. When the cells reached again 80-90% confluency, the cells were splitted again at a ratio of 1:6 (P2) and cultured until they reached 80-90% confluency. Then the cells were splitted a third time (P3). When the cells reached 80-90% confluency, they were irradiated for mitotic inactivation.

#### 2.2.1.2.3 Mitotic inactivation of MEFs with γ-irradiation

The fibroblasts were harvested by trypsinization. Trypsinization was stopped by addition of complete DMEM medium. The cell number was determined and the cells were pelleted by centrifugation at 1,200 rpm for 5 minutes. The cell pellet was suspended in the desired volume of complete DMEM cell culture medium and the cells were irradiated with 7.5 Gray at a dose rate of 0.5 Gray/minute by using a $^{60}$cobalt γ-source. After irradiation, the cell suspension was aliquot into freezing tubes. 10% DMSO were added and frozen in liquid nitrogen. For culturing mESCs, the feeder cells were thawed and plated at a density of $9.1 \times 10^5$ mitotic inactive cells per 50mm cell culture dish.

### 2.2.1.3 Cultivation of mouse embryonic stem cells

D3 and *p53*$^{-/-}$ mouse embryonic stem cells (mESCs) were cultured in GlutaMAX Dulbecco's Modified Eagle Medium (GlutaMAX-DMEM, Gibco) supplemented with 15% fetal bovine serum (PEN-Bio), 100×non-essential amino acids (NEAA, Invitrogen), 1% penicillin/streptomycin (Invitrogen), 0.1mM β-mercaptoethanol (Invitrogen) and 1000 units/ml LIF on feeder cells at 37°C, 5% $CO_2$ and 90% humidity. The medium was changed every day and cells were passaged every second day. For passaging, the cell culture medium was aspirated, and the cells were washed once with PBS (Invitrogen). Then the cells were incubated with trypsin-EDTA (Invitrogen) at 37°C until they were detached from the culture dish. The trypsinization was stopped by addition of five volumes of complete growth medium. The cells were pelleted by centrifugation at 1,200rpm for 2 minutes. The supernatant was aspirated and the cell pellet was suspended in fresh complete medium. $5\times10^4$ cells were transferred into a 50mm cell culture dish containing $9.1\times10^5$ feeder cells prepared as described in section 2.2.1.2.

### 2.2.1.4 Treatment of cells with ionizing radiation and chemicals

Cells were irradiated for 7.5 Gray with a $^{60}$cobalt γ-source at a dose rate of 0.5 Gray/minute in cell culture medium.

Etoposide (Eto) was dissolved in DMSO and used at a final concentration of 50μM. The cells were incubated for 3 hours.

Leptomycin B (LMB) was dissolved in ethanol and used at a final concentration of 2μM. Cells have been serum-starved overnight prior to the addition of LMB and were incubated for 16 hours.

Trichostatin A (TSA) was dissolved in DMSO and used at a final concentration of 1μM. Cells were incubated for 6 hours.

Nicotinamide (NA) was dissolved in dd$H_2O$ and applied at a final concentration of 5mM. Cells were incubated for 6 hours.

Nutlin-3 was dissolved in DMSO and used at a final concentration of 5μM. Cells were incubated as indicated.

### 2.2.1.5 Cell transfection with siRNA

D3 and *p53*$^{-/-}$ mESCs were transiently transfected with siRNA by using MACSfectin$^{TM}$ (Miltenyi Biotec) according to the manufacturers recommendation. For transfection in 24-well plate, 1μg siRNA was diluted in GlutaMAX-DMEM w/o serum or antibiotic in a 1.5 ml polypropylene tube. 2μl of MACSfectin were diluted in another tube. After dilution and homogenized by pipetting, the siRNA and MACSfectin solutions were mixed by pipetting up and down for 3-5 times. The mixture was incubated for 20 minutes at room temperature (RT) to allow the formation of the transfection complex. In the meantime, the cells were trypsinized and collected by centrifugation at 1,200 rpm for 2 minutes. The cell pellets were suspended in complete GlutaMAX-DMEM and the cell number was determined. $1\times10^5$ cells were suspended in 400μl complete GlutaMAX-DMEM and plated into a 24-well plate coated with 0.1% gelatin. 100μl of the transfection mixture were added dropwise to the cell suspension. The culture medium was changed the next day, and cells were harvested 48 hours after transfection.

### 2.2.1.6 Differentiation of mESCs with retinoic acid

mESCs were trypsinized and collected by centrifugation. The cell pellets were suspended in GlutaMAX-DMEM supplemented with 10% fetal bovine serum (PAA), 100×non-essential amino acid (NEAA, Invitrogen), 1% penicillin/streptomycin (Invitrogen), 0.1mM β-mercaptoethanol (Invitrogen) and 1μM all-trans-retinoic acid. $2\times10^5$ *p53*$^{+/+}$ mESCs or $8\times10^4$ *p53*$^{-/-}$ mESCs were seeded in a gelatin-coated 100mm cell culture dish and incubated at 37$^{o}$C in 5% $CO_2$ and 90% humidity for seven days.

The medium was refreshed every second day.

#### 2.2.1.7 Alkaline phosphatase staining

mESCs were cultured on feeder cells in complete GlutaMAX DMEM medium for 48 hours. The culture medium was removed, and the cells were washed with pre-chilled PBS and fixed with 4% PFA in PBS for 20 minutes at room temperature on a shaker. The PFA/PBS solution was removed and the cells were washed three times for 10 minutes at RT with TM buffer (30mM Tris-HCl, 1M maleic acid, 1M NaOH, adjusting pH to 9.0). Then the cells were incubated with freshly prepared staining buffer (5ml TM buffer, 0.75mg Naphtol, 1.25mg Fast Red TR Salt) for 20 minutes at RT on a shaker. The cells were washed twice for 10 minutes with PBS and analyzed under a microscope.

### 2.2.2 Proliferation assays

#### 2.2.2.1 Counting cells with a Neubauer chamber

For counting the cells, the cells were trypsinized with 0.5ml trypsin. Trypsinization was stopped by adding 2ml complete GlutaMAX medium. 10µl of the cell suspension were pipetted into a Neuauer chamber that was covered with a cover slide. The cell number was determined by counting the blue labeled areas (N1-N4; Figure 2.1) under a microscope. The total cell number was calculated with the formula:

$$\text{Cell number per ml} = (N1+N2+N3+N4) \times 10^4/4$$

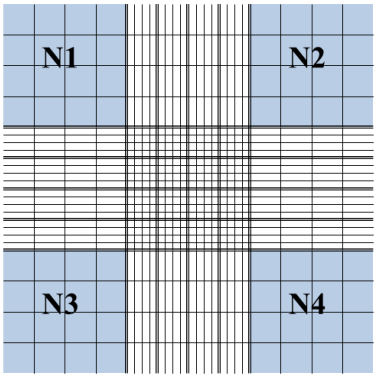


**Figure 2.1 | Schematic picture of a Neuauer chamber.** Shown is the center of a Neubauer chamber with the marks for counting the cells. The cells in the blue areas are counted. The number is then divided by four and multiplied by $1 \times 10^4$. This gives the number of cells per ml.

### 2.2.2.2 MTT assay

After the cells had been incubated for a desired time, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was dissolved in cell culture medium at a concentration of 5mg/ml (MTT solution). 150µl of this MTT solution and 250µl GlutaMAX complete cell culture medium were added to the cells. The cells were incubated at 37$^o$C, in a humidified cell culture incubator for 4 hours. Then the medium was removed, and the cells and the formazan salt were solubilized in 1.5 ml isopropanol. 150µl of this solution were transferred onto a micro-plate and the absorbance of the solution was determined at λ595nm by a micro-plate ELISA reader.

## 2.2.3 Immunofluorescence staining.

For immunofluorescence staining, cover slips were autoclaved and scattered to a 6-well plate. The cover slips were coated with 0.1% gelatin for at least 15 minutes. The gelatin solution was aspirated and feeder cells were plated onto the cover slips. 24 hours after the feeder cells have been seeded, mESCs were plated onto the feeders in GlutaMAX-DMEM complete cell culture medium. 24 hours after plating of the

mESCs, the medium was aspirated and the cover slips with the cells were transferred into a 24-well plate. The slips were washed twice with pre-chilled PBS and fixed with ice-cold acetone/methanol (1:1) for 8 minutes on ice. Then the cover slips were washed three times with cold PBS and blocked for 30 minutes with blocking buffer (1% bovine serum albumin and 1% goat serum in PBS). After blocking, the cells were incubated overnight with primary antibodies. The next day, the cover slips were washed three times with cold PBS and incubated for 30 minutes in the dark with an antibody directed against mouse IgG coupled with Alexa-Fluro-488 (Invitrogen) or with an antibody directed against rabbit IgG coupled with Alexa-Fluro-488 together with Draq5. All reagents were diluted 1:1000 with blocking buffer. After incubation, the cover slips were washed three times with cold PBS and mounted with Hydromount onto microscope slides. The slides were prevented from light until they were analyzed by microscopy.

## 2.2.4 Preparation of cell lysates

Cells were rinsed once with ice-cold PBS and scraped in PBS. The cell suspension was transferred into an Eppendorf tube and cells were collected by centrifugation at 10,000 rpm for 1 minute. The supernatant was removed and the cell pellet was suspended in NP-40 lysis buffer (150mM NaCl, 50mM Tris pH 8.0, 5mM EDTA pH 8.0, 1% NP-40, 1mM PMSF). The samples were incubated on ice for 15 minutes and then centrifuged at 4 ℃ for 10 minutes with 13,300 rpm to pellet insoluble fragments.

### 2.2.4.1 Determination of the protein concentration of cell lysates.

The protein concentration of cellular lysates was determined as described by Bradford (Bradford 1976). To enable the determination of the protein concentration, first a calibration curve was established. Therefore, Roti®-Quant was diluted 1:5 with dd$H_2O$. Then 4µl lysis buffer were mixed with 500µl of the diluted Roti®-Quant

solution and 1, 2 or 4 µl of a solution of 1 mg/ml BSA in $ddH_2O$. 150µl of these mixtures were transferred into a 96-well culture plate. The absorbance of the BSA mixtures was measured at 595nm in a micro-plate reader. For the standard curve, the values of the absorbance and the corresponding BSA protein concentration were plotted in a linear manner. Then 4µl of cell lysate were mixed with 500µl of the diluted Roti®-Quant solution and 150µl of this mixture were transferred into a 96-well plate. The absorbance of protein samples was determined by using a micro-plate reader. The protein concentration was calculated by using the standard curve.

## 2.2.5 SDS PAGE

Prior to the casting of the SDS-PAGE gel, the glass plates were cleaned and dried. A short glass plate was placed in front of a large glass plate, the bottom of the plates was placed accurately flat and the two plates were fixed by a casting frame and transferred to a casting stand. For the separating gel, 4.0ml $ddH_2O$ were mixed with 3.3ml 30% acrylamide mix, 2.5ml 1.5M Tris pH 8.8, 100µl 10% SDS, 100µl 10% APS and 4µl TEMED. The gel mixture was poured between the two glass plates, overlaid with absolute ethanol and allowed to polymerize at room temperature for 30 minutes. After polymerization, the ethanol was removed, the top of the gel was rinsed with $ddH_2O$ and dried with a tissue. The stacking gel consisting of 1.38ml $ddH_2O$, 330µl 30% acrylamide mix, 250µl 1M Tris pH 6.8, 20µl 10% SDS, 20µl 10% APS and 2µl TEMED was poured over the separating gel. A comb was inserted into the stacking gel to create wells. After polymerization, the comb was removed, the gel was inserted into an electrophoresis chamber and the chamber was filled with 1×SDS running buffer (193mM glycine, 24mM Tris and 0.1% SDS).

20-50g of protein were mixed with 20% of a 5×SDS loading buffer (400mM Tris pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol and 0.001% bromophenol blue) and heated to 95°C for 5 minutes. A prestained protein marker, to enable a size

estimation of the separated proteins, and the boiled protein samples were loaded next to each other into the wells of the stacking gel. Electrophoresis was carried out at 130V for approximately 2 hours until the bromophenol blue line reached the bottom of the gel.

For the detection of p53 isoforms, the lysates were mixed with 25% of 4×NuPAGE® LDS Sample Buffer supplemented with 0.1M DTT and heated at 95°C for 5 minutes. 20µg of protein were loaded on pre-casted NuPAGE® 10% Bis-Tris polyacrylamide gels (Invitrogen) and separated by using 1×MOPS Running Buffer (Invitrogen) supplemented with 0.2% antioxidant (Invitrogen) at 120V for 1h 15min.

## 2.2.6 Western blotting and Immunodetection

After electrophoresis, the proteins were transferred onto an Immun-Blot® PVDF (Polyvinylidene difluoride) membrane using a Bio-Rad semi-dry Trans-Blot® Turbo™ Transfer System. The Immun-Blot® PVDF membrane was rinsed with absolute ethanol until the membrane became translucent. The ethanol was removed and the membrane was equilibrated in Trans-Blot Turbo Transfer Buffer for approximately 2-3 minutes. Two transfer stacks were immersed in a soaking tray containing 50-70ml of transfer buffer for 2-3 minutes. The transfer sandwiches were assembled according to the scheme provided in Figure 2.2. Excess transfer buffer was carefully removed by inverting the cassette base with the assembled stack.

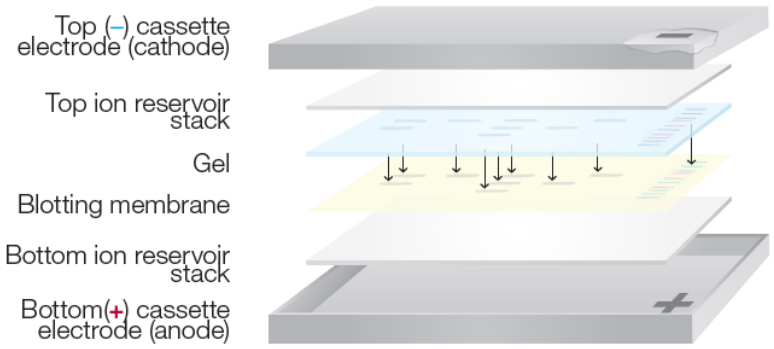


**Figure 2.2 | Layering of the western blot transfer pack.** A membrane was laid in the center of the base of the ion reservoir stack (anode stack). Then the gel was aligned on the membrane. Air bubbles between the gel and the membrane were removed with a blot roller. The second ion reservoir stack (cathode stack) was then place onto the gel. Air bubbles in the assembled transfer stack were removed by a blot roller so that consistent contact was allowed between the layers. (Source: http://www.bio-rad.com/)

Then the lid of the cassette was placed onto the base and the transfer was performed with 1.3A and up to 25V for 16 minutes. After the transfer, the membranes were immersed in 2% ink in TBS/0.2% Tween20 (TBST, 50mM Tris-Cl pH 7.6, 150mM NaCl and 0.2% Tween20) for 10 minutes to see whether the membrane was free of air bubbles. The membrane was rinsed twice with TBST and blocked with blocking buffer (5% skimmed milk powder in TBST (w/v)) for 1 hour. After blocking, the membrane was incubated overnight with the primary antibody diluted in 5% BSA/TBS (w/v). The next day, the membrane was washed three times for 5 minutes with TBST and incubated for 1-2 hours with an HRP-coupled secondary antibody diluted in 5% BSA/TBS (w/v). After incubation with the secondary antibody, the membrane was washed twice for 5 minutes in TBST and once for 10 minute in TBS. A mixture of equal volumes of ECL solution I and II (Pierce) was distributed evenly across the membrane and incubated at room temperature for I minute. The membrane was wrapped into cling film and exposed against an X-ray film (GE Healthcare) in the dark rom. The exposed X-ray film was developed by an X-ray film processor.

## 2.2.7 Cell Fractionation

For performing cell fractionation, the cells were washed with ice-cold PBS, scraped from the culture dish and collected by centrifugation The supernatant was aspirated and the cell pellet was suspended in four packed cell volumes homogenization buffer (10mM HEPES pH 7.4, 50mM NaCl, 0.5M sucrose, 0.5% Triton X-100, 1mM PMSF), incubated on ice for 5 minutes and collected by centrifugation at 4$^{o}$C with 1,000rpm for 10 minutes. The supernatant that contained the cytoplasmic fraction was carefully transferred into a new 1.5ml Eppendorf tube and kept on ice. The nuclei were washed twice with ice-cold PBS and suspended in the same volume of lysis buffer that was used to release the cytoplasmic fraction, disrupted by sonication with 400W for 20 cycles (1 sec on/1 sec off) and kept on ice. Both fractions were used for performing SDS-PAGE (See section 2.2.5 and 2.2.6) or stored at -20$^{o}$C.

Alternatively, cells were washed, scraped into PBS and pelleted by centrifugation at 4$^{o}$C with 1,200 rpm for 5 minutes. The supernatant was aspirated and the cell pellet was re-suspended in a small volume of homogenization buffer (10mM HEPES pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT, 1×EDTA-free Protease Inhibitor Cocktail) and incubated on ice for 5 minutes. The nuclei were released by 15-25 strokes with a homogenizer and collected by centrifugation at 4$^{o}$C with 300g for 5 minutes in. The supernatant containing cytoplasmic fraction was transfered into a new 1.5ml Eppendorf tube and kept on ice. The nuclei were suspended in 0.25M sucrose buffer (0.25M Sucrose, 10mM $MgCl_2$, and 1×EDTA-free Protease Inhibitor Cocktail) and layered onto a cushion of 0.88M sucrose buffer (0.88M Sucrose, 0.5mM $MgCl_2$, 1×EDTA-free Protease Inhibitor Cocktail). The nuclei were collected by centrifugation at 4℃ with 2,800g for 10 minutes. The supernatant was removed and the nuclei were lysed in lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 5mM EDTA, 1% NP-40, 1mM PMSF, 1×EDTA-free Protease Inhibitor Cocktail). Both fractions were used for performing SDS-PAGE (See section 2.2.5) or stored at -20$^{o}$C.

## 2.2.8 Two-dimensional SDS PAGE

Two-dimensional SDS PAGE was performed to separate proteins both by their molecular weight and by their isoelectric point (Figure 2.3).

The medium was aspirated from the cells, the cells were washed once with cold PBS scraped from the culture dish, transferred to a reaction tube and collected by centrifugation. The supernatant was removed, the cells were suspended in Urea Lysis buffer (8.5M Urea, 4% CHAPS, 50mM Tris pH 8.0, 1mM PMSF) and disrupted by sonication for 10 seconds. The lysate was cleared by centrifugation at 4 ℃ for 20 min with 13,300 rpm. The supernatant was transferred to a new 1.5ml Eppendorf tube and the protein concentration was determined as described above (See section 2.2.4). 1mg of the protein was diluted with an equal volume of 2×IEF buffer (5M Urea, 2M Thio-urea, 65mM CHAPS, 4mM tributylphosmin and 1% Carrier-ampholyte pH 3-11 NL). The volume was adjusted to 335µl with Rehydration buffer (5M Urea, 2M Thio-urea, 65mM CHAPS, 2mM tributylphosmin, 0.5% Carrier-ampholyte pH 3-11 NL). 5µl 0.8% (w/v) Brilliant Blue G in rehydration buffer were added, the sample was loaded onto a 18 cm Immobiline DryStrip pH 3-11 NL and allowed to soak overnight.

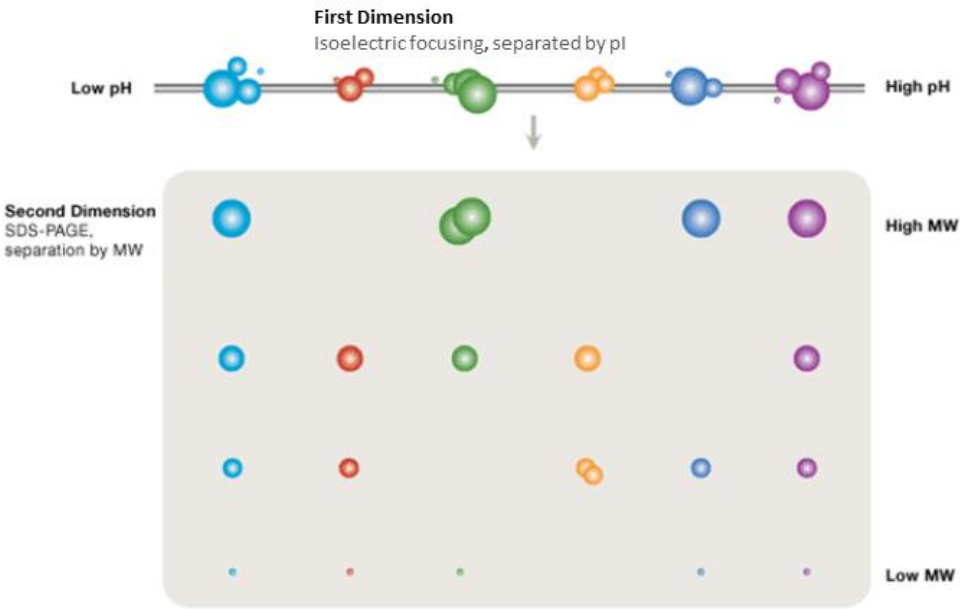

**Figure 2.3 | Schematic picture of Two-D SDS PAGE.** The proteins were first separated by their isoelectric point (pI, 1st dimension). Then proteins were further separated by their molecular weight (MW, 2nd dimension). The balls in distinct color represent proteins with distinct pIs. The size of the colored balls represents the different MWs of the proteins, big balls mean high MW proteins, and small balls mean low MW proteins.

The next day, the protein was separated by isoelectric focusing. The stripe was placed in an Ettan IPGphor II Isoelectric Focusing system (GE Healthcare) and covered with mineral oil. Isoelectric focusing was performed at 25°C and 200V for 3.5 hours, at 500V for 3.5 hours, at 1000V for 3.5 hours, at a gradient ramp up to 8000V for 1 hour and at 8000V for 11 hours. After separation in the first dimension by isoelectric focussing, the strips were rinsed with $ddH_2O$ to remove excess mineral oil. The IEF gel was equilibrated in equilibration buffer (50mM Tris pH 8.8, 6M urea, 30% Glycerol, 2% SDS) plus 65mM DTT for 15 min and alkylated for 15 min in 20mg/ml iodoacetamide in equilibration buffer containing 0.03% Commassie Brilliant Blue G in $ddH_2O$. 12.5% SDS polyacrylamide gels (For 100ml solution: 33ml $ddH_2O$, 40ml 30% acrylamide mix, 25ml 1.5M Tris pH 8.8, 1000µl 10% SDS, 1000µl 10% APS and 40µl TEMED) were casted in the PROTEAN Plus Multi-Casting Chamber up to 1 cm below the end of the small glass plate and overlaid with isopropanol. After the polymerization of the acrylamide mixture, the isopropanol was removed and the top of the gel was rinsed with $ddH_2O$. The equilibrated gel strips from the isoelectric focusing were carefully laid onto the top of the polymerized polyacrylamide gel and immersed in 0.8% Commassie Brilliant Blue G in 1% (w/v) agarose. After the solidification of the agarose, the gels were transferred into the Ettan DALT large vertical system and the electrophoresis chamber was filled with a Tris-glycine buffer (193mM glycine, 24mM Tris and 0.1% SDS). Electrophoresis was performed at 2W per gel for 6 hours. After electrophoresis, the proteins were transferred onto a PVDF membrane by Western Blotting and detected by immunodetection as described (See section 2.2.6).

## 2.2.9 Sucrose gradient centrifugation

Sucrose gradient centrifugation was performed to separate proteins and protein complexes under native condition. In the sucrose gradient, the particles travel through the gradient until they reach the layer where the density of the particles matches that of the surrounding sucrose.

For the gradient, sucrose was dissolved in 25mM Tris pH 7.4, 50mM NaCl, 1mM PMSF to achieve a sucrose concentration of 10% and 40%. The two sucrose solutions were poured into a gradient mixer connected to a mini-pump that transferred the mixture into a polyallomer centrifuge tube. The gradient was kept on ice until it was used.

To prepare the lysate, the medium was aspirated and the cells were washed twice with ice-cold PBS, scraped into PBS and collected by centrifugation at 13,300 rpm for 10 seconds at 4℃. The cell pellet was suspended in lysis buffer (50mM Tris pH 7.4, 20mM NaCl, 10mM $MgCl_2$, 0.5% NP-40, 5mM ATP, 1mM DTT, 1mM PMSF, 40U/ml DNase I, 10mM N-ethylmaleimide, 10mM 1,10-Phenanthtroline and 1×Phosphostop), homogenized by pushing it 3 times through a 26G needle and incubated on ice for 30 min. The protein extract was cleared by centrifugation at $4^oC$ with 13,300 rpm for 15 min. The protein concentration was determined and 2mg protein were loaded onto the sucrose gradient. The sucrose gradient was placed into a SW 50.1 rotor (Beckman) and centrifuged at 4 ℃ for 18 h with 37,000 rpm. After centrifugation, fractions were collected and analyzed by SDS PAGE and Western Blotting (see section 2.2.5 and 2.2.6).

## 2.2.10 RNA sequencing

### 2.2.10. 1 Extraction of total RNA from eukaryotic cells

Total RNA was prepared using TRIzol according to the manufacturer’s instructions

Cells were washed with PBS, scraped in PBS and pelleted by centrifugation. TRIzol was added to the pellet and the mixture was homogenized by vortexing. The Homogenized samples were kept at room temperature for 5 minutes to permit complete lysis. 0.2ml chloroform were added per ml TRIzol, vortexed and incubated at room temperature for 3 minutes. The samples were centrifuged at 10,000 rpm for 15 minutes to allow the different phases to separate. The aqueous phase that contained the RNA was carefully transfered into a fresh reaction tube, mixed with 0.5ml isopropanol per ml TRIzol and incubated on ice for 10 minutes to precipitate the RNA. The RNA was collected by centrifuged at 4℃ with 10,000 rpm for 15 minutes. The supernatant was removed and the RNA was washed twice with 1ml 75% ethanol. Finally, the RNA was dissolved in RNase free water and handed over to our sequencing facility.

### 2.2.10. 1 Analysis of the sequencing results

The sequencing resulted in more than sixty-seven million reads per sample each of which being 50 nucleotides long with a mean Phred quality score over 35 and reading errors smaller than two to the minus thirty-five.

The reads where then mapped against the mouse genome M37 database using the TOPHAT software (Trapnell et al., 2010). To see whether the fragment sequences belong to distinct exons, the BOWTIE software was applied. Finally, gene expression was determined using the HTSeq software (Anders and Huber, 2010). This software counted for each gene the number of reads. The software DESeq was then used to normalize the data (Figure 2.4; Anders and Huber, 2010).

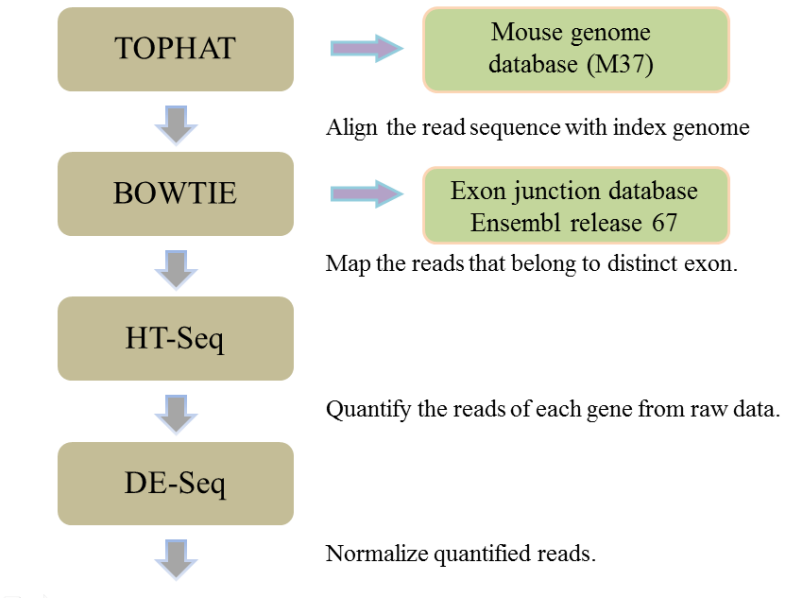


**Figure 2.4 | Scheme of RNA sequencing analysis.** The reads were mapped against the mouse genome M37 database by applying the TOPHAT software. The exon junstions were determined using the BOWTIE software and the Ensembl release 67 database. Gene expression was determined with the HTSeq software and differential gene expression was determined using the DESeq software.

### 2.2.11 Chromatin-immunoprecipitation and polymerase chain reaction

Chromatin-immunoprecipitation (ChIP) was performed to detect p53 bound to promoters of target genes.

#### 2.2.11.1 Chromatin-immunoprecipitation (ChIP)

Proteins and DNA were cross-linked by adding formaldehyde to the culture medium to a final concentration (f.c.) of 1% and incubation for 10 min at room temperature (RT) on a rocking platform. The reaction was stopped by addition of glycine (0.125M f.c.) and incubation for 5 min at RT. The media was removed and the plates were washed twice with ice-cold PBS. The supernatant was removed, the cells were scraped from the dish, collected by centrifugation and washed with cold PBS

containing 1mM PMSF. The cells were suspended in 3 volumes lysis buffer (5mM HEPES pH 8, 85mM KCl, 0.5% NP40, 1mM PMSF, 1µg/ml aproptinin, 1µg/ml leupeptin) and incubated on ice for 10 minutes. The nuclei were pelleted at 4℃ for 5 min with 5000 rpm, and suspended in nuclei lysis buffer (50mM Tris pH 8.1, 10mM EDTA pH 8.0, 1% SDS, 1mM PMSF, 1µg/ml aproptinin, 1µg/ml leupeptin) and incubated for 10 min on ice. 200µl of the nucleic lysate was transferred into a 0.5ml thin wall tube. The lysate was sonicated (peak incidence power 140W, duty factor 5.0%, 200Hz and 900seconds) to achieve an average length of the chromatin of about 400bp. After sonication, the lysate nucleic lysate was cleared by centrifugation at 4℃ for 10 min with 13,000 rpm. The supernatant was transferred into a fresh reaction tube and diluted 5 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA pH 8.1, 16.7mM Tris-Cl pH 8.0, 167mM NaCl, 1mM PMSF, 1µg/ml aproptinin, 1µg/ml leupeptin). The samples were pre-cleared with a mixture of sonicated salmon sperm DNA, BSA and protein A agarose for 30 min at 4℃ with end-over-end rotation. The protein A agarose was pelleted at 4℃ for 5 minutes with 7,200 rpm and the supernatant was transferred to a fresh reaction tube. 10% of the supernatant were saved for the input control. The remaining lysate was divided into two parts. To one part, IgG was added to control for the antibody specificity. To the second part, the anti-p53 antibody CM5 was added. The samples were incubated at 4℃ overnight with end-over-end rotation. The next morning, 30µl of a 1:1 slurry of sonicated salmon sperm DNA, protein A agarose and BSA in TBS (50mM Tris-Cl pH7.5, 150mM NaCl) were added and incubated for 1 h at 4℃ with agitation. The precipitates were pelleted by centrifugation at 4℃ for 3 min with 7200 rpm and consecutively washed once with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA pH 8.1, 20mM Tris-Cl, pH 8.0), twice with high salt wash buffer (0.1% SDS, 1% Triton X 100, 2mM EDTA, pH 8.1, 20mM Tris-Cl, pH 8.0, 500mM NaCl), once with LiCl wash buffer (10mM Tris-Cl pH 8.0, 250mM LiCl, 1% NP40, 1% deoxycholic acid, 1mM EDTA pH 8.1) and twice with TE buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.1). The samples were briefly centrifuged at 4℃ for 3 minutes with

7,200 rpm, any traces of buffer were removed and the antibody/protein/DNA complexes were eluted twice with 150 µl freshly prepared IP elution buffer (100mM $NaHCO_3$, 1% SDS) for 15 minutes on a mixer at maximal speed. The supernatants from both elutions were combined and the sample was centrifuged at room temperature for 3 minutes with 13,200 rpm to remove any traces of the protein A agarose. The supernatant was transferred to a fresh reaction tube.

The crosslinks were removed from the sample by incubation with RNase A at 65 °C overnight in the presence of 0.3M NaCl (f.c.). 2½ volumes ethanol were added and the DNA was precipitated at -80 °C for 1 hour and collected by centrifugation at 4 °C for 20 minutes with 14,000 rpm. The supernatant was aspirated, the pellet was air-dried and suspended in 10mM EDTA pH 8.1, 40mM Tris-Cl pH 6.5. 150 µg/ml proteinase K were added and the sample was incubated for 2 h at 45 °C to digest any remaining protein. 175 µl TE buffer (10mM Tris-Cl pH7.5, 1mM EDTA) were added and the sample was extracted once with 300 µl phenol/chloroform/isoamyl alcohol and twice with 300 µl chloroform/isoamyl alcohol. 30 µl of 5M NaCl, 5 µg tRNA and 5 µg Roti-Pink and 800 µl ethanol were added and the DNA was precipitated at -20 °C overnight. The DNA was collected by centrifugation at 4 °C for 20 minutes with 14,000 rpm and air-dried. The DNA pellet was dissolved in 50 µl $ddH_2O$ and analyzed by PCR.

## 2.2.11.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was applied to amplify DNA fragments. This technique makes use of the principle that primers that are associated with a DNA fragment can be extended by the DNA polymerase Repeats of a denaturing, annealing and extension step allow the amplification of a DNA fragment from one copy to billion copies.

For the PCR reaction, a template, in this case DNA purified from ChIP was mixed with 1 µl of a forward and a reverse primer (each 50 µg/µl), 0.5 µl dNTPs (10mM),

GoTaq polymerase (Promega) and 2µl 10×GoTaq polymerase buffer and adjusted to 20µl with $ddH_2O$. The DNA was first denatured at 95°C for 2 minutes, followed by several cycles of a denaturing step (95°C, 30 sec), an annealing step (55°C, 20 sec), and an extension step (72°C, 30 sec). The number of cycles depends on the abundance of the template. After the polymerization reaction, the PCR product was analyzed by Agarose gel electrophoresis.

### 2.2.11.3 Agarose gel electrophoresis

1g agarose was boiled in 100ml 1×TAE buffer (40mM Tris pH 7.2, 20mM NaAc, 1mM EDTA) until the agarose was completely dissolved. Ethidium bromide was added to a final concentration of 0.1µg/ml when the agarose solution was a bit cooled down. The solution was poured into a horizontal gel base. A comb was inserted into the agarose solution in order to produce wells where the DNA samples could be loaded. After solidification, the gel was inserted into a horizontal electrophoresis chamber and the chamber was filled with 1×TAE buffer until the whole gel was immersed in the buffer. The PCR products were mixed with 6×loading dye and carefully pipetted into the wells. A 2-log DNA-ladder was also applied next to the samples to allow an estimation of the size of the DNA fragment. Electrophoresis was performed at 160V until the colored front reached the middle or 2/3 of the gel. The DNA fragments were visualized under UV light and photographed.

## 2.2.12 Extraction of RNA from eukaryotic cells and quantitative PCR (qPCR)

Total RNA was purified by using the RNeasy Kit (Qiagen) according to the manufacturer's instruction: Cells were washed twice with ice-cold PBS, scraped into PBS and collected by centrifugation at 4℃ for 10 seconds with 13,300 rpm. The cell pellet was lysed in 600µl RLT buffer supplemented with 143mM β-mercaptoethanol.

The lysate was homogenized by passing it through a 20G needle fitted to a syringe. After homogenization, the lysate was mixed with the same volume of 70% ethanol and transferred onto an RNeasy spin column. The column was centrifuged with 10,000 rpm for 15 sec at RT. The flow-through was removed and the column was successively washed once with 700 µl RW1 buffer and twice with 500 µl RPE buffer. The flow-through was discarded and the column was centrifuged for an additional minute to remove residual wash buffer. The RNA was eluted with 40 µl RNase-free dd$H_2O$ and collected by centrifugation with 10,000 rpm for one minute. The concentration of the RNA was determined with a NanoDrop spectrophotometer.

The optical density (OD) of the nucleic acid solution was measured at 260nm, 280nm and 230nm respectively. A unit of $OD_{260}$ corresponds to 40 µg/ml of single-stranded RNA calculated by the Beer-Lambert equation. The ratio of $OD_{260}/OD_{280}$ was used to estimate the purity of RNA. A value of 2.0 indicates an RNA solution that is free of protein, phenol and other contaminants.

For cDNA synthesis, 1 µg RNA was adjusted to 7 µl with nuclease free water. 1 µl rRNasin (20-40u/µl), 1 µl 10×DNase I buffer and 1 µl DNase I were added and the sample was incubated for 30 minutes at 37$^{o}$C to remove genomic DNA. The activity of DNase I was stopped by adding 1 µl of a DNase stop solution followed by incubation at 65$^{o}$C for 15 minutes. For first strand synthesis, 1 µl of random primers (200ng/µl) were added and incubated at 70$^{o}$C for 5 minutes. A mixture of 0.5 µl of dNTPs (10mM each), 1 µl of M-MLV RT (200u/µl), 4 µl of M-MLV RT buffer and 4.5 µl of nuclease free water were then added and the reaction was incubated successively at 37$^{o}$C for 10 minutes, 42$^{o}$C for 40 minutes, and 70$^{o}$C for 10 minutes. After reverse transcription, the cDNA was diluted in 100 µl nuclease free water and kept in -20$^{o}$C. For control, RNA was treated identically yet the reverse transcriptase was omitted from the reaction.

To control the quality of the reverse transcription, a PCR was performed with primers

for the house-keeping gene RiBPO (See section 2.2.12.6) and analyzed by agarose-gel electrophoresis (See section 2.2.12.7).

qRT-PCR was performed to determine the relative amount of different RNAs. 2 µl of the cDNA were mixed with 10 µl 2×SYBER GREEN Real-time Master Mix, 1 µl each of a gene-specific forward and reverse primer (50 ng/µl), and 4 µl nuclease free water and processed and analyzed by the ABI StepOnePlus System. The program was set according to the scheme shown in Figure 2.5.

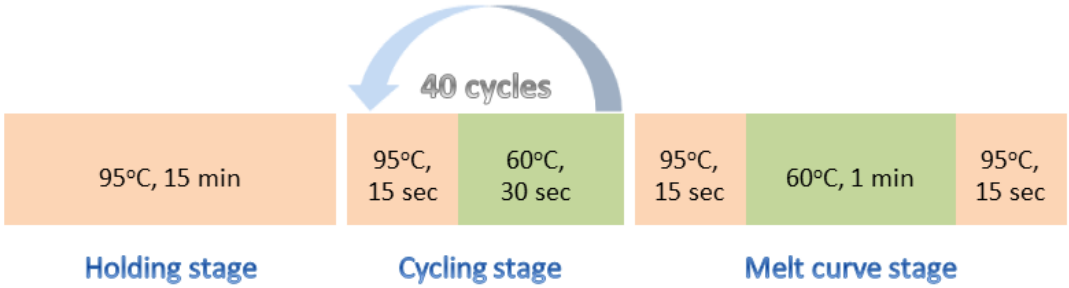


**Figure 2.5 | Scheme of the qRT-PCR cycling procedure.** The reaction starts with heating the samples for 15 min to 95°C followed by 40 cycles or denaturation, annealing and extention (95°C for 15 sec for denaturing and 60°C for 30 sec for annealing and extension). The third step is the melt curve and holding stage.

All PCR reactions were performed in duplicates and with a control where no template has been added. A PCR reaction with primers for RiBPO was performed for internal control. A threshold was set to subtract the background. When the fluorescence intensity reached the threshold, the level of transcripts was recorded as the cycle threshold ($C_T$). The relative amount of each gene was calculated by the $\Delta\Delta C_T$ method:

$\Delta C_T$ (treated group) = $C_T$ (treated group target) - $C_T$ (treated group reference (RiBPO))

$\Delta C_T$ (control) = $C_T$ (control target) - $C_T$ (control reference (RiBPO))

Then, the ΔΔCT between control and the sample was calculated:

$$\Delta\Delta C_T = \Delta C_T \text{ (control) } - \Delta C_T \text{ (treated group)}$$

Finally, the normalized ratio of the expression of a target gene was determined by the formula: $2^{-\Delta\Delta C_T}$

# 3 RESULTS

## 3.1 p53 is localized in the nucleus in mouse embryonic stem cells.

The p53 protein is one of most well-known tumor suppressor protein and plays a crucial functions in somatic cells including induction of cell cycle arrest or apoptosis in response to DNA damage (Riley et al., 2008). However, despite of its anti-proliferative effect, p53 is reported to be highly expressed in mouse embryonic stem cells (mESCs) (Sabapathy et al., 1997; Solozobova and Blattner, 2010), a cell type that proliferates faster than somatic cells because of a shortened G1 phase (Becker et al., 2006). Before I started my investigations, I set out to confirm the previous observations that stem cells proliferate faster than somatic cells and that p53 is present in a higher amount in mESCs than in differentiated cells. I lysed mESCs and mESCs that had been differentiated by incubation with retinoic acid for seven days and monitored abundance of p53 by Western blotting. For control, I also employed murine embryonic fibroblasts. To control for the specificity of the p53 antibody, I included *p53*$^{-/-}$ counterparts of the above mentioned cell types.

As shown in figure 3.1A, the p53 protein was highly abundant in ESCs, and its abundance was strongly decreased in differentiated mESCs. In mouse embryonic fibroblasts (MEFs), p53 was even undetectable under these conditions. Hybridization with an antibody targeted against the stem cell marker Oct3/4 shows that the stemness character was present in mESCs and strongly decreased after incubation with retinoic acid to a level that was comparable to the MEFs (Figure 3.1A).

In order to investigate whether mESCs proliferate at a higher rate than somatic cells, I plated $5\times10^4$ mESCs per well into a 6-well plate, and to some of the cells, I added 1μM (f.c.) retinoic acid at the time of plating to induce differentiation. For control, I

employed MEFs. Three days after plating, I determined the cell number.

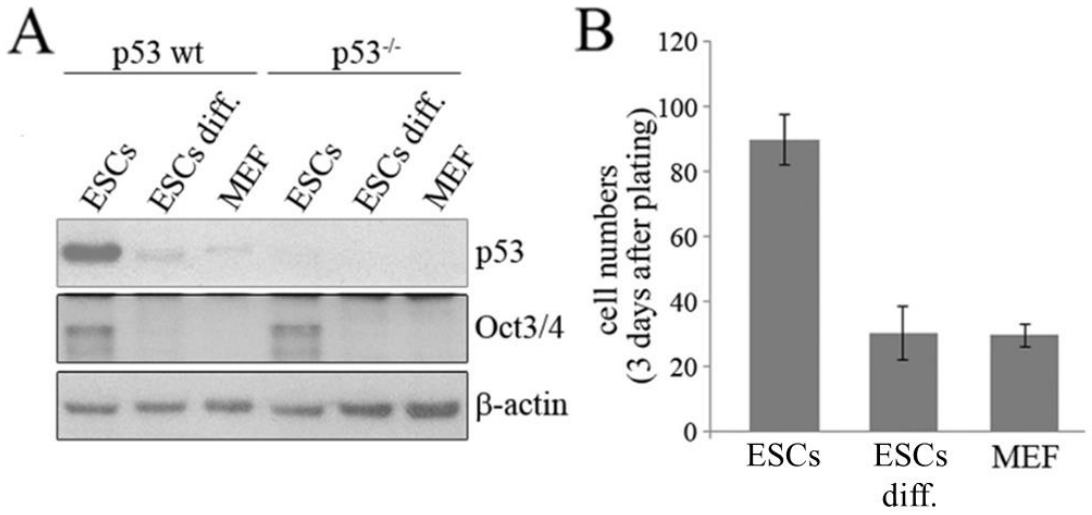


**Figure 3.1 | p53 is highly expressed in mouse embryonic stem cells. A.** murine embryonic fibroblasts (ESCs), mESCs that had been differentiated with 1μM retinoic acid for seven days (ESCs diff.), mouse embryonic fibroblasts (MEF) and their p53-deficient counterparts were lysed and the cell lysate was separated by SDS PAGE. The separated proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk/PBST for 1 hour, incubated with the anti-p53 antibody 1C12 at 4°C overnight, washed 3 times with PBST and incubated with an HRP-coupled anti mouse antibody for 1 hour at room temperature. The membrane was washed twice with TBST and once with TBS. A mixture of an equal volumes of ECL solution I and II was distributed evenly over the membrane, and incubated for 1 minute. The membrane was wrapped in cling film and exposed against an X-ray film (GE Healthcare). Subsequent hybridizations with antibodies targeted against Oct3/4 to control for stemness; and against β-actin, for loading control, and p53 were performed as described for p53. **B.** mESCs and MEFs were plated at a density of $5 \times 10^4$ cells/well in a 6-well plate. 1μM Retinoic acid (f.c.) was added to one well of mESCs (mESCs diff.) at the time of plating. Cells were counted three days after plating. The graph shows mean values and standard deviations of three independent experiments.

As shown in figure 3.1B, the number of ESCs was almost three times higher than that of those ESCs that had been induced to differentiate (ESCs diff.) or of MEFs (Figure 3.1B). Thus, mESCs proliferate significantly faster than differentiated cells despite the high amount of p53.

As p53 is highly abundant in mESCs but the cells proliferate much faster than MEFs, for example, which have low amounts of p53 (Figure 3.1A), this raised the issue how

stem cells can proliferate fast regardless of the presence of a high amount of the p53 proteins, that is highly anti-proliferative, at least in differentiated cells (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990). Previous publications argued that mESCs can proliferate fast because of a cytoplasmic localization of p53 in stem cells (Aladjem et al., 1998; Han et al., 2008; Solozobova et al., 2009) that would interfere with its activities as a transcription factor. Since the localization of p53 might be crucial for its activity, I investigated whether p53 is indeed sequestered in the cytoplasm in mESCs.

I first performed immunofluorescence staining for p53 of mESCs. I plated mESCs on feeder cells on and cultured them for two days. To control for the specificity of the antibodies, I also plated p53-deficient mESCs. I then fixed the cells, washed them with PBS and blocked them with 1% bovine serum albumin and 1% goat serum in PBS prior to the incubation with several different anti-p53 antibodies. Among the antibodies that I used was the anti-p53 antibody Pab421 that was used in previous investigations (Aladjem et al., 1998; Solozobova et al., 2009). To control for stemness, I also included an antibody against Nanog (Mitsui et al., 2003). For some slides, I omitted the first antibody, to control for the specificity of the secondary antibody. The next day, I washed the cover slips with PBS and incubated them with antibodies directed against mouse or rabbit IgG coupled to a fluorescent dye together with Draq5. After incubation, I washed the cover slips, mounted them onto microscope slides and analyzed them with a confocal microscope. In agreement with previous studies (Aladjem et al., 1998; Solozobova et al., 2009), I observed a signal in the cytoplasmic compartment in wild type mESCs when I applied the anti-p53 antibody Pab421 (Figure 3.2). However, I also observed a signal of similar intensity in the cytoplasm of *p53*$^{-/-}$ mESCs which are devoid of the p53 protein. Obviously the antibody Pab421 recognizes a protein in mESCs that is not p53. In contrast, when I used the CM5 or the 1C12 anti-p53 antibody, I observed the majority of staining in the nuclear compartment of the stem cells. Moreover, I did not observe any staining in *p53*$^{-/-}$ mESCs when I employed the anti-p53 antibody 1C12, and only a weak staining when

I used the antibody CM5 (Figure 3.2). This result indicates that p53 may be localized in the nucleus in stem cells. Thus, the cytoplasmic appearance that was seen when the antibody Pab421 was used could eventually be an antibody-artifact.

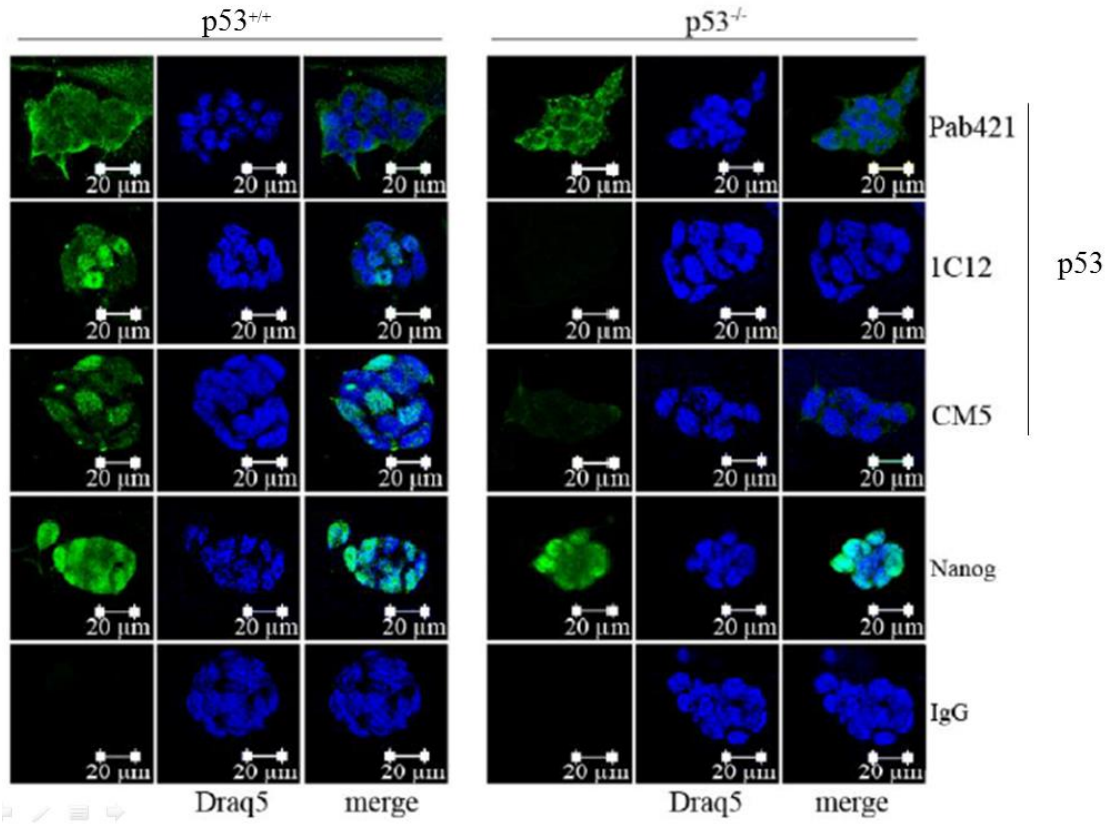


**Figure 3.2 | p53 is localized in the nucleus in mouse embryonic stem cells.** mESCs (*p53*$^{+/+}$) and their p53-deficient derivative (*p53*$^{-/-}$) were grown on feeder cells on coverslips, fixed with ice-cold acetone/methanol, blocked with 1% bovine serum albumin and 1% goat serum in PBS and incubated with the indicated antibodies diluted in 1% bovine serum albumin and 1% goat serum in PBS (Pab421: 1:200; 1C12: 1:2000; CM5: 1:1000; Nanog: 1:500; IgG: 1:1000. After primary antibody incubation and washing, the cells were incubated for 30 minutes in the dark with antibodies directed against mouse or rabbit IgG coupled with Alexa-Fluro-488, diluted 1:1,000 (shown in green) together with Draq5 diluted 1:3,000 (shown in blue). Images were analysed on a Leica LSM microscope.

To confirm the result from the immunofluorescence analysis, I fractionated mESCs into cytoplasmic and nuclear lysate and determined the amount of p53 in the two fractions. Apart from wild-type and *p53*$^{-/-}$ mESCs, I also included mESCs that had been treated with retinoic acid to induce differentiation. To fractionate the cells, I suspended the cell pellet in a low salt homogenization buffer, after I had collected

them by centrifugation, incubated the samples shortly on ice to disrupt the cellular membrane and pelleted the nuclei by centrifugation. The supernatant after this centrifugation represented the cytoplasmic fraction. I purified the nuclei further by centrifugation through a sucrose cushion and disrupted them by applying a high salt lysis buffer. I then analyzed both fractions by SDS PAGE and western blotting. To compare the different anti-p53 antibodies, I had prepared four identical membranes onto which I had loaded an equal number of cells of the different cell types. Then I hybridized each of the membranes with a different anti-p53 antibody (Pab421, Pab246, CM5, 1C12). To control the purity of each fraction, I also monitored the GAPDH protein that is merely expressed in the cytoplasm and the Histone H3 protein that is merely expressed in the nucleus.

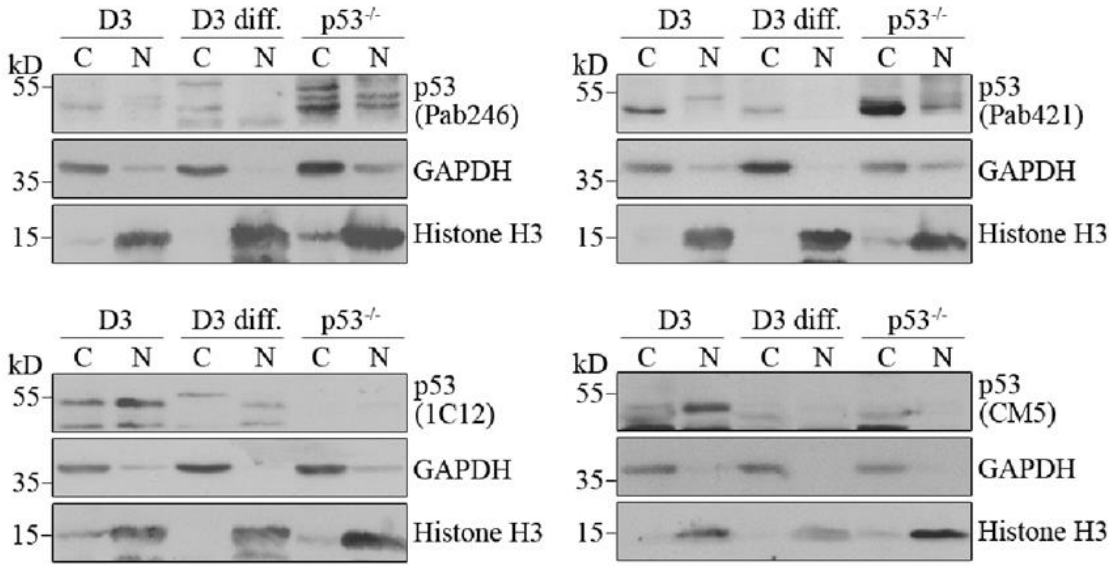


**Figure 3.3 | The majority of p53 is nuclear in mESCs.** *p53*$^{+/+}$ mESCs (D3), p53$^{-/-}$ mESCs, and *p53*$^{+/+}$ mESCs that had been differentiated by incubation with RA (D3 diff.) were harvested, washed with ice-cold PBS, scraped from the culture dish and collected by centrifugation The supernatant was aspirated and the cell pellet was suspended in four packed cell volumes homogenization buffer, incubated on ice for 5 minutes and collected by centrifugation. The supernatant that contained the cytoplasmic fraction was transferred into a new reaction tube. The nuclei were washed twice with ice-cold PBS, suspended in the same volume of lysis buffer that was used to release the nuclear fraction and disrupted by sonication. Lysates corresponding to equal amounts of cells were analysed by SDS PAGE and western blotting. Four identical membranes were prepared and each was incubated with a different anti-p53 antibody (1C12, CM5, Pab246, Pab421) as described in the legend to figure 3.1. Hybridization with GAPDH and Histone H3 was employed to monitor the efficiency of fractionation.

In consistency with the result from the immunofluorescence analysis, the anti-p53 antibody Pab421 showed a strong signal in the cytoplasmic fractions from p53 positive stem cells and differentiated cells (Figure 3.3). However, there was an even stronger signal also present in p53 negative cells. This was also the case for the Pab246 antibody that was used in a previous report (Han et al., 2008). The antibodies 1C12 and CM5 gave a clear signal at approximately 53 kDa that was only detectable in p53 positive cells. The majority of this signal was in the nuclear fraction (Figure 3.3), confirming the result obtained from immunofluorescence analysis. However, I also observed that some p53 protein was present in the cytoplasmic fraction, indicating that the p53 protein is present both in the nuclear and in the cytoplasmic compartment in stem cells (Figure 3.3). In differentiated cells, I could detect p53 only in the nuclear fraction. This was probably due to the low amount of p53 in this cell type and the poor sensitivity of this assay.

In order to obtain further evidence that p53 is nuclear in stem cells, I treated cells with the CRM1 inhibitor Leptomycin B (LMB). LMB inhibits the shuttling of Mdm2 between cytoplasm and nucleus resulting in increased p53 abundance in the nucleus of differentiated cells (Roth et al., 1998). Thus, if the p53 protein would be purely localized in the cytoplasm in stem cells, p53 should not accumulate in the nucleus after addition of LMB. To test this prediction, I first starved the cells for 16 hours by incubating the cells in culture medium without fetal bovine serum. I then changed the medium and incubated the cells overnight in complete cell culture medium and 20nM LMB. The next morning, I harvested the cells. I took an aliquot of the cells to analyze abundance of p53 in the whole cell lysate and fractionated the remaining cells into cytoplasmic and nuclear lysate. I then performed SDS PAGE and western blotting for further analysis. I prepared two identical membranes and hybridized these membranes with the anti-p53 antibodies 1C12 or CM5.

In figure 3.4 it is shown that the p53 protein accumulated dramatically in the nucleus of mESCs (Figure 3.4). In addition, p53 accumulated in the cytoplasm of stem cells

(Figure 3.4). This was not the case for e.g. PARC or Nanog, which were used to monitor stemness and efficient fractionation of the cells (Figure 3.4).

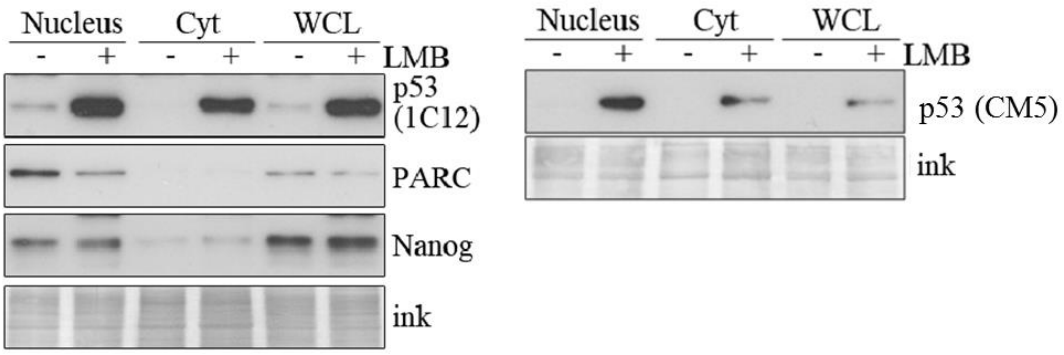


**Figure 3.4 | The p53 protein accumulates in stem cells after inhibition of nuclear export by Leptomycin B.** mESCs were starved with serum free medium for 16 hours, then the medium was changed and the cells were cultured overnight in complete GlutaMAX DMEM containing 20nM LMB. The cells were harvested and an aliquot of the cells was reserved for whole cell lysate while the rest of the cells were separated into a cytoplasmic and nuclear fraction. Therefore, the cells were pelleted at 4$^{o}$C for 5 minutes with 1,200 rpm. The cell pellet was suspended in homogenization buffer and incubated on ice for 5 minutes. The nuclei were released by 15 strokes with a homogenizer and collected by centrifugation. The supernatant contained the cytoplasmic fraction. The nuclei were purified by centrifugation through a sucrose cushion and lysed. The protein concentration of all the samples was determined and 50 µg of the cellular lysates were analyzed by SDS PAGE and western blotting as described in the legend to figure 3.1. p53 was detected by hybridization with the anti-p53 antibody 1C12 (diluted 1:10,000) and CM5 (diluted 1:2,000). Hybridization of the membranes with PARC was used to monitor fractionation. Hybridization with Nanog was performed to control for the pluripotency of the stem cells. Membranes were stained with ink to control for equal loading of the membranes. Cyt, cytoplasm; WCL, whole cell lysate.

## 3.2 The Anti-proliferative activity of p53 is compromised in mESCs.

Since a majority of p53 is obviously nuclear also in stem cells, a different localization of p53 in stem cells and differentiated cells cannot account for the high proliferation rate of stem cells in the presence of these high amounts of p53. However, if p53

would be inhibited in other ways, this could explain the high proliferation rate of mESCs. In order to test whether p53 is active in stem cells, I made use of the observation that cells that possess wild type p53 usually proliferate faster than their p53-negative counterpart (see e.g. (Li et al., 2012). If I would not see such a reduction in stem cells, this would be a good indication, that at least the anti-proliferative activity of p53 would be compromised in stem cells. I therefore monitored the proliferation of p53-positive mESCs and their p53-negative counterpart. I plated the stem cells on feeder cells and counted the cells every day. For control I plated feeder cells alone. To control for the authenticity of the result, I also monitored the proliferation of p53-positive and p53-negative MEFs, and of stem cells that were induced to differentiate by adding all-trans-retinoic acid.

As shown in Figure 3.5A, p53 positive stem cells proliferated as fast as p53 negative cells (Figure 3.5A). In contrast to the mESCs and in consistency with the reported work (Li et al., 2012), p53-positive MEFs proliferated much slower than the p53-negative MEFs (Figure 3.5A). Also, p53-positive differentiated cells proliferated slower than their *p53*$^{-/-}$ counterpart (Figure 3.5A). These results suggest that the variation in the proliferation rate in the presence and absence of p53 is an attribute of differentiated cells but not of stem cells.

To confirm the result that the proliferation of stem cells is not affected by the presence of p53, I performed an MTT assay. The MTT assay is a colorimetric assay that measures the reduction of 3-(4, 5-dimethythialzol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into the insoluble formazan salt by the mitochondrial succinate dehydrogenase. As this reduction of MTT is accompanied by a change in the color, the conversion can be monitored spectrophotometrically. Since reduction of MTT only occurs in metabolically active and thus in living cells, it can be used to determine the relative amount of living cells (Heeg et al., 1985).

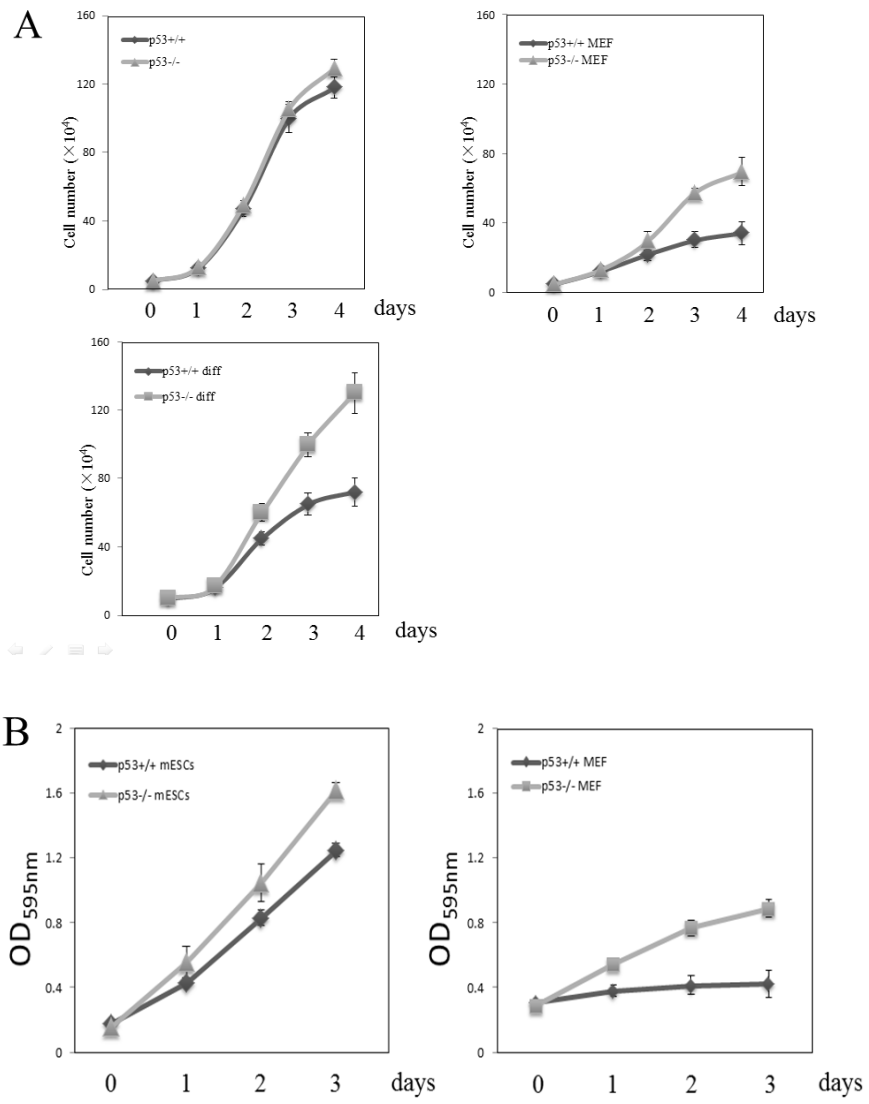


**Figure 3.5 | The anti-proliferative activity of p53 is compromised in mESCs. A.** Wild-type mESCs ($p53^{+/+}$), their p53-deficient derivative ($p53^{-/-}$), mouse embryonic fibroblasts ($p53^{+/+}$ MEF) and their p53-deficient counterpart ($p53^{-/-}$ MEF) were plated at a density of $5\times10^4$ cells/well in a 6-well plate. Differentiated mESCs (mESCs diff.), and the corresponding $p53^{-/-}$ cells were plated at a density of $1\times10^5$ cells/well. At the time of plating, 1μM (f.c.) retinoic acid was added to differentiate the cells. All cells were counted each day for three days. The graph shows mean values and standard deviations of three independent experiments. **B.** $p53^{+/+}$ mESCs and their corresponding $p53^{-/-}$ derivative ($p53^{-/-}$ mESCs) were plated at a density of $5\times10^4$ cells/well in a 6-well plate on feeder cells. $p53^{+/+}$ MEFs and their p53-deficient counterpart ($p53^{-/-}$ MEFs) were plated at a density of $5\times10^4$ cells/well in a 6-well plate. At one, two and three days after plating, the medium was aspirated and 125μl MTT solution (5mg/ml) and 250μl complete growth medium were added and the cells were incubated for 4 hrs at 37℃ and 6% $CO_2$. Then the medium was aspirated and 125ul isopropanol were added to each well and vortexed to release the formazan. 100μl of the formazan solution were diluted 1:10 with isopropanol and 150ul of this solution were transferred into a 96-well plate. The absorbance was determined at 595 nm. The graph shows mean values and standard deviations of three independent experiments.

For the MTT assay, I seeded $5x10^4$ p53-positive ESCs and their p53-negative counterpart (*p53*$^{-/-}$) per well in a 6-well plate and determined the MTT conversion each day. To control the authenticity of this assay, I also measured the MTT conversion in p53-positive (*p53*$^{+/+}$ MEF) and p53-negative MEF (*p53*$^{-/-}$ MEF).

In figure 3.5 B, it is shown that the conversion of MTT is a bit lower in *p53*$^{+/+}$ stem cells than in *p53*$^{-/-}$ stem cells (Figure 3.5B). However, the difference in the MTT conversion between *p53*$^{+/+}$ stem cells and *p53*$^{-/-}$ stem cells was much smaller than that of *p53*$^{+/+}$ and *p53*$^{-/-}$ MEFs (Figure 3.5B). Thus this experiment confirms the result from counting the cells that the effect of p53 on the proliferation of stem cells is insignificant, which is in contrast to the effect of p53 on the proliferation of differentiated cells.

The above shown results indicate that the anti-proliferative activity of p53 is compromised in mESCs. However, these results could also be due to changes that may have occurred in the p53-negative mESCs in order to adapt to the absence of p53. To see whether the similar proliferation rate of p53-positive and p53-negative mESCs may indeed be caused by adaptive changes in *p53*$^{-/-}$ stem cells or whether it really reflects a compromised function of p53 in mESCs, I downregulated p53 in mESCs and in mESCs that had been differentiated by incubation with retinoic acid. Seventy-two hours after siRNA transfection, I monitored cell proliferation by the MTT assay and downregulation of p53 by western blotting.

As shown in figure 3.6A, differentiated cells clearly proliferated faster when p53 was downregulated. Such a difference in cell proliferation was, however, not seen when p53 was downregulated in mESCs (Figure 3.6A). Here, the cells proliferated even slightly slower, although the difference was not statistically significant. The Western blot shown in part B shows that p53 was downregulated to a similar extent in the stem cells and the differentiated cells (Figure 3.6B).

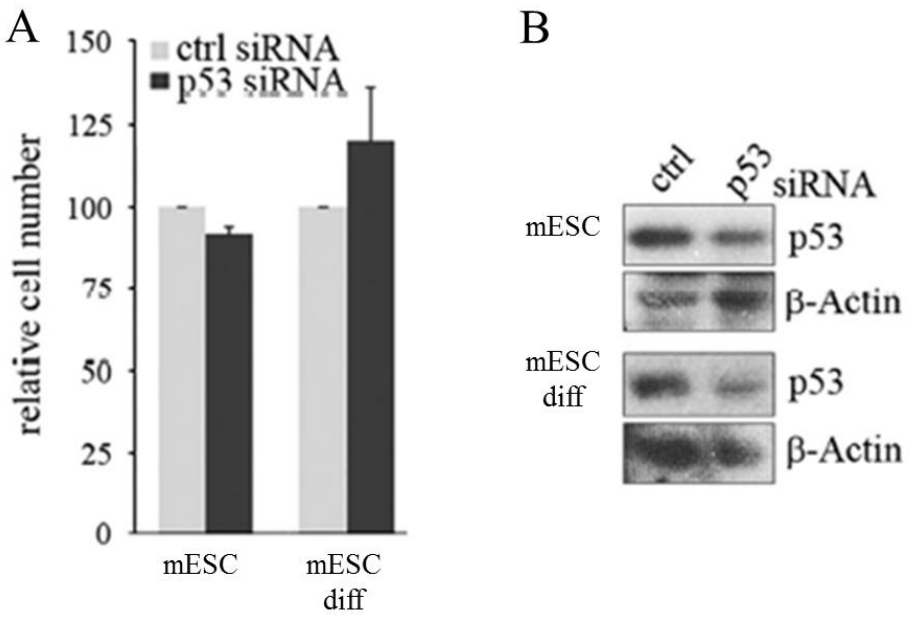


**Figure 3.6 | Transient downregulation of p53 did not change the proliferation of mESCs.** mESCs and mESCs that had been differentiated by incubation with retinoic acid for seven days (mESCs diff.) were transfected with a siRNA targeted against p53 or with a control siRNA in triplicates. **A.** Seventy-two hours after transfection, 125ul MTT solution (5mg/ml) and 250µl DMEM were added to two wells of the triplicate and the cells were incubated for 4 hrs. Then the medium was aspirated and 125µl isopropanol were added to each well and vortexed thoroughly to release the formazan. 100µl of the dissolved formazan solution were diluted 1:10 with isopropanol and 150µl of this solution were transferred into a 96-well plate. The absorbance of the diluted formazan solution was measured at 595 nm. The graph shows mean values and error bars of two (mESCs diff.) or three (mESCs) independent experiments. **B.** The third sample of the triplicate was lysed in NP40 lysis. 30 µg of the lysate were separated by an SDS-PAGE gel and further analyzed by western blotting. p53 was detected by hybridizing the membrane with the 1C12 anti-p53 antibody as described in the legend to figure 3.1. Hybridization with β-Actin was performed for loading control

Finally, I treated the cells with Nutlin in order to see whether p53 is active in stem cells. Nutlin is a cis-imidazoline analogy that binds to the p53-binding pocket of Mdm2 and inhibits the interaction between MDM2 and p53. In the presence of Nutlin, p53 is released from Mdm2-mediated control which leads to the stabilization and activation of p53 (Vassilev et al., 2004) independent of post-translational modifications that occur e.g. in response to DNA damage. If p53 is active in mESCs, the administration of Nutlin should strongly reduce the proliferation of mESCs as it does in more differentiated cells (Zauli et al., 2007).

To investigate whether p53 is active in mESCs, I incubated mESCs and mESCs that had been differentiated by incubation with retinoic acid for seven days with 5 µM Nutlin. 72 hours after addition of Nutlin, I monitored the cell density by MTT assay. For control, I also measured the cell density by MTT assay in *p53*$^{-/-}$ mESCs (*p53*$^{-/-}$) and their differentiated derivatives (*p53*$^{-/-}$ diff).

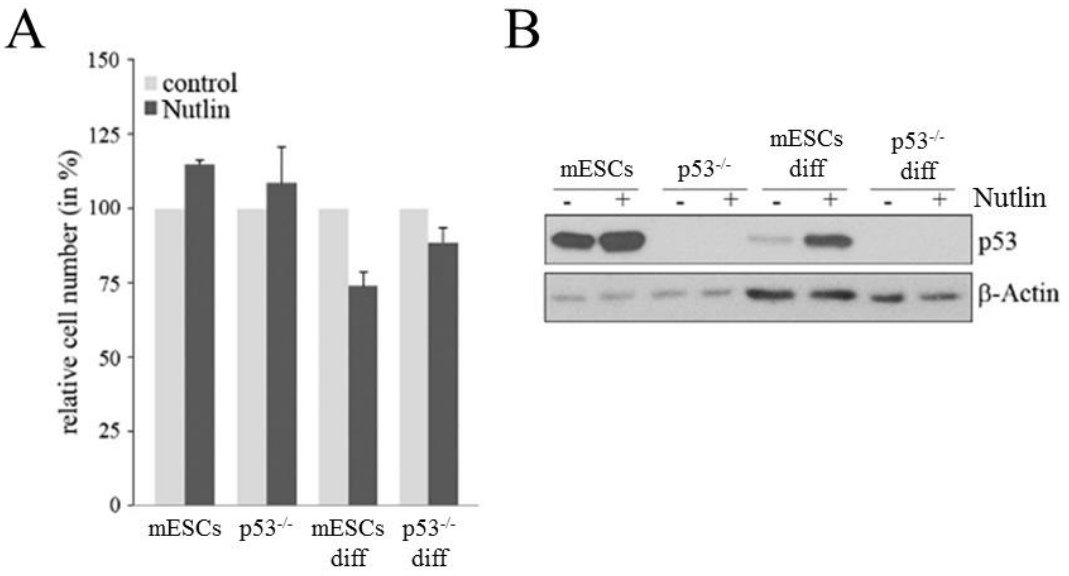


**Figure 3.7 | Induction of p53 by Nutlin had no effect on the proliferation of mESCs. A.** mESCs, mESCs that had been differentiated with retinoic acid (mESCs diff.), and their p53-deficient counterparts (*p53*$^{-/-}$ and *p53*$^{-/-}$ diff) were plated in quadruplicates and treated with 5 µM Nutlin or with DMSO for control. Seventy-two hours after plating, relative cell proliferation was assessed from triplicates by MTT assay as described in the legend to figure 3.6A. The graph shows mean values and error bars of two independent experiments. Relative cell numbers of mock treated cells were set to 100%. **B.** The remaining part of the quadruple was lysed thirty-two hours after Nutlin treatment and abundance of p53 was assessed by Western blotting as described in the legend to figure 3.1 using the 1C12 anti-p53 antibody diluted 1:10,000. Hybridization with β-Actin (diluted 1:1,000) was performed for loading control.

As shown in figure 3.7A, the treatment of differentiated cells with Nutlin obviously reduced cell proliferation (mESCs diff.) when p53 was present, which is consistent with the report by Zauli and colleagues (Zauli et al., 2007). In contrast, the treatment of mESCs with Nutlin did not reduce their proliferation (Figure 3.7A). Part B of the figure shows that treatment of the cells increased p53 abundance both in stem cells and in differentiated cells (Figure 3.7B).

All these results support the concept that the anti-proliferative activity of p53 is compromised in mESCs.

## 3.3 p53 is modified at lysine 379, serine 15 and serine 392 in response to DNA damage in mESCs, but not under normal culture condition.

Since I found that the anti-proliferative activity of p53 is compromised in mESCs, I wondered which mechanism could impede p53's activity. As p53's activity is tightly controlled in somatic cells by a variety of posttranslational modifications (PTM) (Bode and Dong, 2004; Boehme and Blattner, 2009; Dai and Gu, 2010), I wondered whether the impaired anti-proliferative activity of p53 in stem cells could also be due to PTMs and whether the PTMs of p53 are different in mESCs and differentiated cells. To investigate this possibility, I monitored p53's PTM in mESCs and differentiated cells by using commercially available antibodies against p53 phosphorylated at serine 6 (S6), at serine 15 (S15) and at serine 392 (S392) as well as against p53 acetylated at lysine 379 (K379). I investigated modifications at these sites since these PTMs were reported to be crucial for p53's activity (Dumaz and Meek, 1999; Higashimoto et al., 2000; Keller and Lu, 2002; Sagakuchi et al., 1998). I therefore plated mESCs and mESCs that had been differentiated for seven days with retinoic acid. For positive control, I subjected a part of the stem cells and differentiated cells to ionizing radiation since DNA damage has been shown to increase p53's PTMs at these sites (Dumaz and Meek, 1999; Higashimoto et al., 2000; Keller and Lu, 2002; Sagakuchi et al., 1998). To control for the specificity of the antibodies, I included the p53-negative counterparts of the stem cells and differentiated cells. Two hours after irradiation, I lysed the cells and monitored p53 modifications as well as total levels of p53 by western blotting.

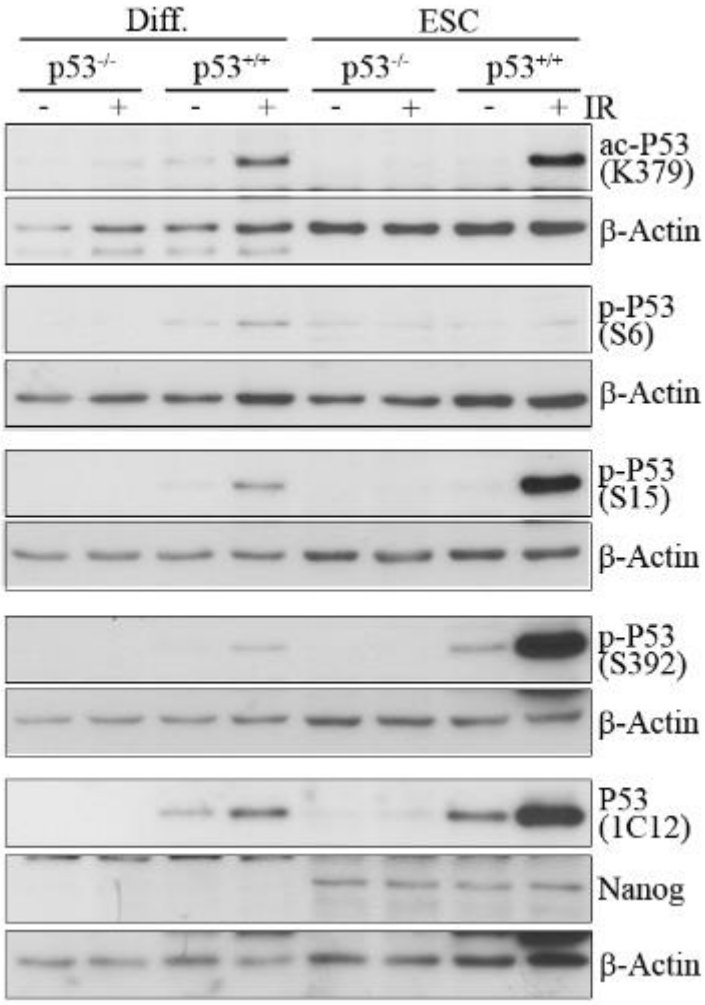


**Figure 3.8 | p53 is modified at lysine 379, serine 15 and serine 392 in response to DNA damage.** mESCs, mESCs that had been differentiated with retinoic acid for 7 days (Diff.) and their p53-negative counterparts were irradiated with 7 Gray or left unirradiated. Two hours after irradiation, cells were harvested and the cell number was determined. Then the cells were lysed at 1million cells/100µl lysis buffer. Five identical membranes were prepared by separating 30µl of cell lysate per lane by SDS-PAGE and transferring the proteins onto a PVDF membrane. The membranes were hybridized as described in the legend to figure 3.1 with antibodies directed against phosphorylated p53 (S6, diluted 1:1,000), phosphorylated p53 (S15, diluted 1:3,000), phosphorylated 53 (S392, diluted 1:3,000), acetylated p53 (K379, diluted 1:3,000) or against pan-p53 (1C12, diluted 1:10,000). Hybridization with Nanog (diluted 1:2,000) was performed to control for stemness and with β-Actin (diluted 1:1,000) to allow for the comparison of the loading of the different membranes. Western Blots were developed by ECL.

As shown in figure 3.8, p53 was acetylated at K379 and phosphorylated at S15 and S392 after DNA damage. However, apart from the anti-phosphorylated p53 antibody

at serine 392, which gave a very weak signal also for non-irradiated mESCs, none of the antibodies recognized p53 of undamaged cells, neither in stem cells nor in differentiated cells (Figure 3.8). None of these antibodies gave signals in p53 negative cells, proofing their specificity (Figure 3.8).

## 3.4 A fraction of p53 with a neutral pI exists exclusively in mouse embryonic stem cells.

All the antibodies against modified p53 that were used in figure 3.8 were raised against sites of p53 that are modified in response to DNA damage., However, as shown in figures 3.5 to 3.7, p53's activity was compromised in ESCs under normal growth conditions. Thus, if the PTMs of p53 differ between stem cells and differentiated cells, these modifications may be different from those that are added to p53 after genotoxic stress. Valeriya Solozobova from our lab, had already observed that mESCs have a fraction of p53 with a neutral pI that is absent in p53 from

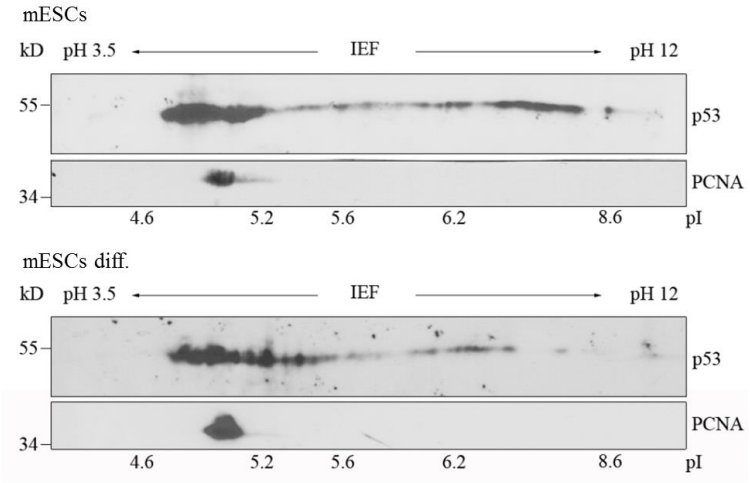


**Figure 3.9 | A fraction of p53 with a neutral pI exists exclusively in stem cells.** Lysates of mESCs and mESCs that had been differentiated with retinoic acid for 7 days (mESCs Diff.) were separated by two-dimensional gel electrophoresis and blotted. Abundance of p53 was determined by incubation with the 1C12 antibody. Hybridization with an antibody targeted against PCNA (proliferating cell nuclear antigen) was performed for control. (Courtesy of Valeria Solozobova)

differentiated cells (see Figure 3.9). I thought that this difference could be important for stem cell function and therefore I elaborated further on this discovery. I was particularly interested whether this alteration is due to changes in the phosphorylation or acetylation of p53 since these modifications have a strong impact on p53 function (Boehme and Blattner, 2009). To find out whether p53 is acetylated in mESCs, I treated the cells with a combination of nicotinamide (NA) and trichostatin A (TSA) prior to harvesting the cells. Both compounds inhibit histone deacetylases, the major deacetylating enzymes in the cell (Bitterman et al., 2002; Yoshida et al., 1990). These inhibitors furthermore strongly enhance p53 acetylation in differentiated cells (Luo et al., 2001; Terui et al., 2003). In order to find out whether p53 is phosphorylated in mESCs, I treated the cell lysate with λ-phosphatase. I then separated the cell lysates by 2D-gel electrophoresis and monitored p53 abundance by western blotting. To control for the activity of λ-phosphatase, I irradiated mESCs and differentiated cells with ionising irradiation, which strongly increases p53 phosphorylation at serine 15 (Dumaz et al., 1999; see also figure 3.10). I prepared cellular lysate from these cells and treated a part of the lysate under the same conditions that I used for treating the cell lysate that I then analysed by 2D-western blotting.

As shown in figure 3.10A, treatment of the lysate of stem cells with λ-phosphatase shifted a fraction of p53 beyond a pI of 8.6. In samples that had not been treated with the phosphatase, there was much less p53 with a pI greater than 8.6 (Figure 3.10A). This result shows that p53 in stem cells is constitutively phosphorylated. For differentiated cells, I obtained a similar result. Here, the charge of p53 was also changed after phosphatase treatment. Here, a fraction of p53 appeared with a pI between 5.6 and 8.6 that is absent in the control cells. However, no p53 with a pI beyond 8.6 was detected in these samples. In fact, p53 from differentiated cells that had been treated with phosphatase resembled untreated p53 from stem cells (Figure 3.10A). Part B of this figure shows a strong decrease in p53 phosphorylation when the samples had been treated with λ-phosphatase prior to gel electrophoresis demonstrating that the treatment with the phosphatase indeed removed phosphate

groups from p53 under the applied conditions A strong signal for the stem cell marker Oct3/4 shows that the mESCs that were used for this experiment had maintained their stem cell properties (Figure 3.10B).

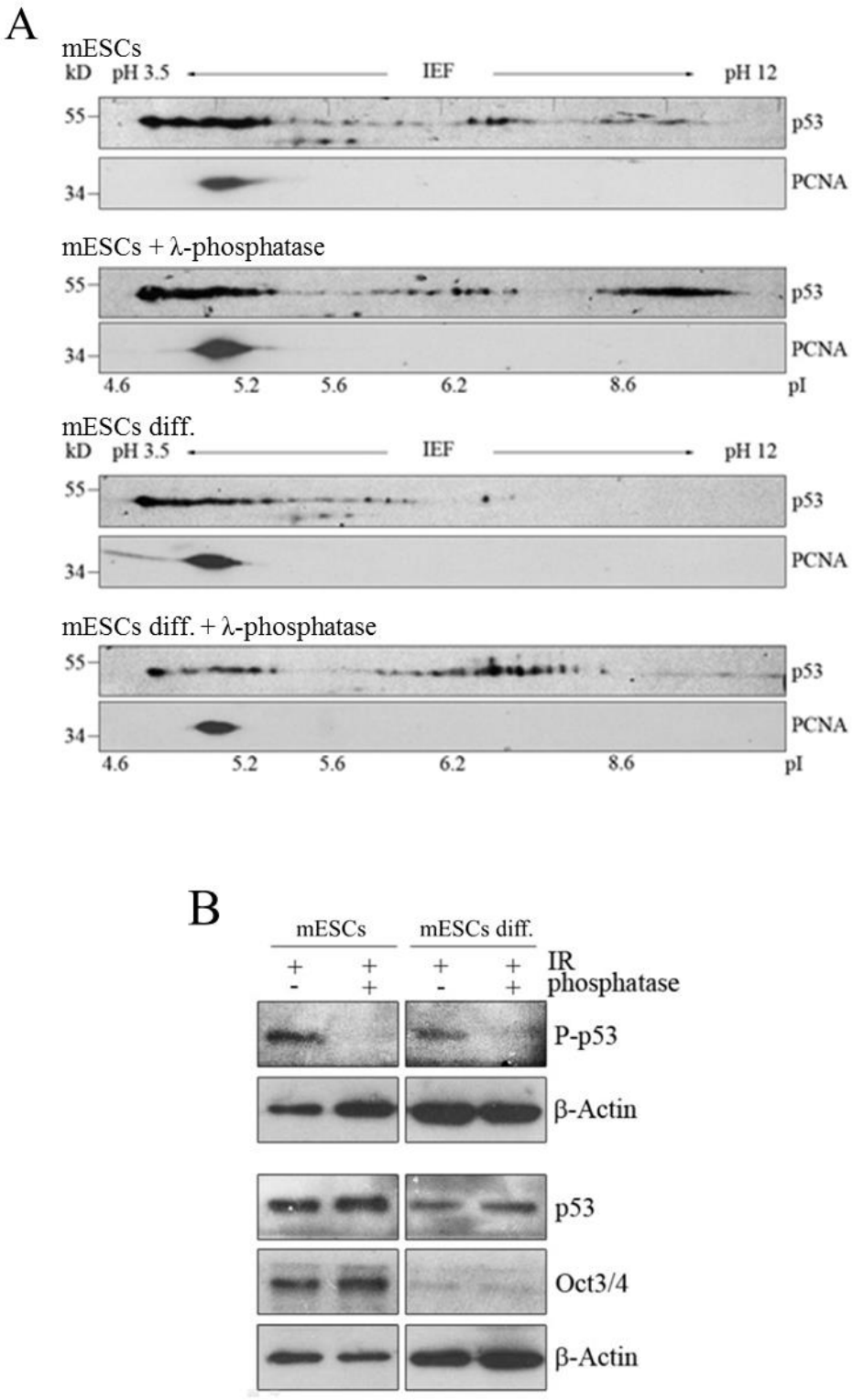

**Figure 3.10 | p53 is constitutively phosphorylated in mESCs.** mESC and mESCs that had been differentiated with retinoic acid for 7 days (mESC Diff.) were harvested, suspended in phosphatase buffer and sonicated. Where indicated, cells were irradiated with 7.5 Gray and harvested 30 minutes after irradiation. Lambda phosphatase was added at a concentration of 200 units per 100 µg of protein (or cells were left without phosphatase for control) and incubated for 30 minutes at 30 ℃. The proteins were TCA-precipitated, the pellet was suspended in Urea Lysis buffer and the lysate was cleared by centrifugation. **A.** 1mg of the protein was diluted with an equal volume of 2×IEF buffer, loaded onto an 18 cm Immobiline DryStrip pH 3-11 NL and incubated until all the sample solution was sucked by the strip. Isoelectric focusing (IEF) was performed at 200V for 3.5 hours, at 500V for 3.5 hours, at 1000V for 3.5 hours, at a gradient up to 8000V for 1 hour and at 8000V for 11 hours. After IEF, the gel was equilibrated and alkylated and layered on top of an SDS-PAGE gel. After electrophoresis, the proteins were transferred onto a PVDF membrane and detected by immunodetection as described in the legend to figure 3.1. **B.** 50µg of protein were separated by SDS-PAGE and transferred onto a PVDF membrane. Determination of the amount of phosphorylated p53 (antibody diluted 1:1,000), total p53 (1C12 diluted 1:10,000), Oct 3/4 (antibody diluted 1:2,000) to control for stemness, and β-Actin (antibody diluted, 1:1,000) for loading control, was performed as described in the legend to figure 3.1.

Treatment of mESCs with a combination of TSA and NA strongly increased the negative charge of p53 from stem cells resulting in the complete removal of the fraction of p53 with a neutral pI (Figure 3.11A). In differentiated cells, there was also a tendency to increase the fraction pf p53 with a pI between 4.6 and 5.2 (Figure 3.11). The effect was, however, not as obvious as in stem cells. Part B of this figure shows that the treatment with TSA and NA increased p53 acetylation under the applied conditions in stem cells and differentiated cells (Figure 3.11B). A strong signal for the stem cell marker Oct3/4 demonstrates the pluripotency of the mESCs that were used for this experiment (Figure 3.11B).

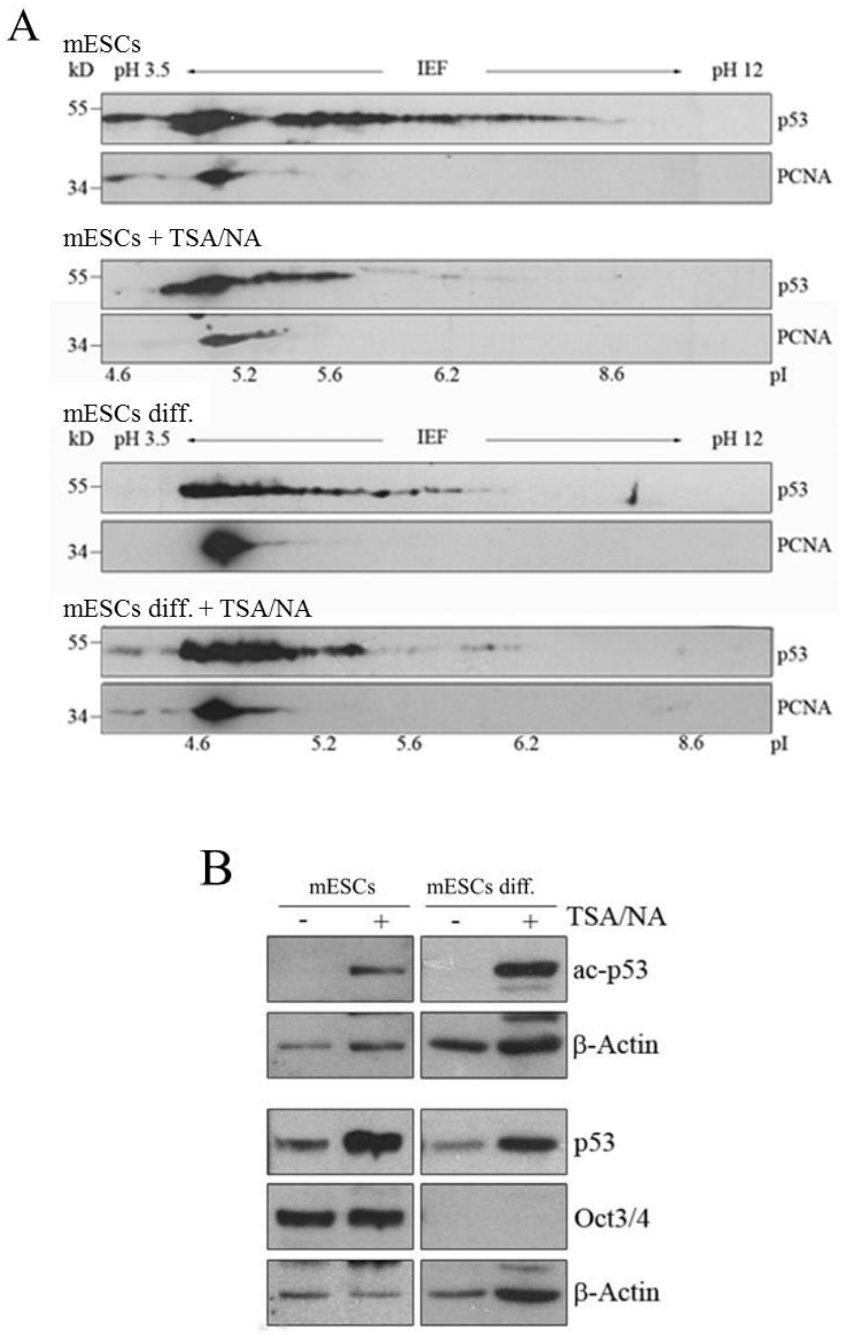


**Figure 3.11 | p53 is constitutively deacetylated in stem cells.** mESCs and mESCs that had been differentiated with retinoic acid for 7 days (mESCs Diff.) were treated with 1 μM trichostatin A and 5 mM nicotinamide for 6 hours. **A.** The cells were suspended in Urea Lysis buffer and processed as described in the legend to figure 3.10A. **B.** 50 μg of proteins were separated by SDS-PAGE gels and transferred onto a PVDF membrane. Determination of phosphorylated p53 (antibody diluted 1:1,000), total p53 (1C12, diluted 1:10,000), Oct 3/4 (antibody diluted 1:2,000) to control for stemness, and β-Actin (antibody diluted, 1:1,000) for loading control, was determined as described in the legend to figure 3.1.

## 3.5 p53 is present in a large protein complex in mESCs that contains the inhibitory protein MDMX.

Another possibility to achieve inactivation of p53 is by its association with inhibitory proteins (Boehme and Blattner, 2009). In order to get an idea whether p53 is associated with different proteins in mESCs and differentiated cells, I performed sucrose gradient centrifugation and monitored p53's abundance in the different fractions. Sucrose gradient centrifugation is a method by which proteins and protein complexes can be separated by their molecular weight under native conditions. After layering the protein lysate onto the sucrose gradient, the gradient is centrifuged. During this centrifugation step, proteins and protein complexes travel through the sucrose gradient until they arrive at the layer where the density of the sucrose gradient matches with their molecular weight. After this centrifugation step, fractions can be taken and analysed for the presence of the protein of interest. However, while this method is well appropriate to monitor changes in the size of proteins or protein complexes, it does not tell which proteins are in the complex unless further analytical tools such as western blotting are performed. To investigate whether p53 from mESCs and from differentiated cells may be in different protein complexes (and thus associated with different proteins), I used mESCs and differentiated cells, lysed the cells in a mild buffer to prevent the dissolution of the complexes and loaded the cleared cellular lysate onto a sucrose gradient. After centrifugation, I collected fourteen fractions and analysed these fractions by western blotting.

Most interestingly, hardly any p53 protein existed as monomers or dimers both in stem cells and in differentiated cells. In fact, most p53 existed in protein complexes larger than 660 kDa. Thus, p53 is almost exclusively associated with other proteins and protein complexes. In contrast to my expectations, I could not see a difference in the distribution of p53 between mESCs and differentiated cells (Figure 3.12). Thus, there is no obvious difference in the size of the complexes which contain p53 in

mESCs and differentiated cells. This, however, does not rule out the possibility that there is a difference in individual proteins in these complexes. In differentiated cells, p53 activity is mainly inhibited by MDM2 and MDMX (Shvarts et al., 1996; Haupt et al., 1996). I therefore wondered whether there is a difference in the binding of MDM2 or MDMX to p53 between mESCs and differentiated cells. I reasoned that if MDM2 or MDMX are associated with p53 in stem cells or differentiated cells, then they should co-elute with p53 from sucrose gradients. I therefore analysed the fractions from the sucrose gradient also for the presence of MDM2 and MDMX.

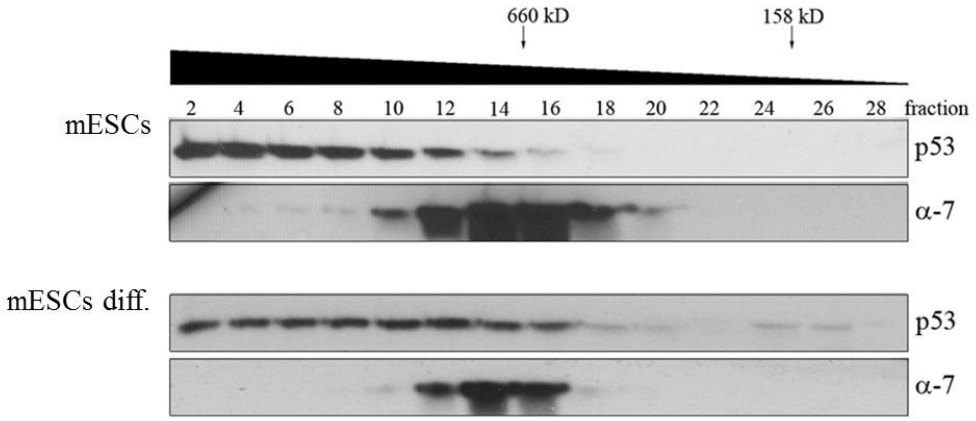


**Figure 3.12 | The distribution of p53 after sucrose density centrifugation is not altered after differentiation.** mESCs and mESCs that had been differentiated by incubation with retinoic acid for 7 days (mESCs diff.) were lysed in a mild lysis buffer and cleared by centrifugation. 2mg of the lysate were loaded onto a 10-40% sucrose gradient and centrifuged for 18 h. After centrifugation, fractions were collected and further analyzed by SDS PAGE and western blot. The abundance of p53 was detected by hybridization with the anti-p53 antibody 1C12 (diluted 1:0,000) as described in the legend to figure 3.1. Hybridization with α-7 (MCP72, diluted 1:2,000) was performed for internal control.

As shown in figure 3.13, MDMX eluted in exactly the same fractions from the sucrose gradient as p53, indicating that these two proteins could associate with each other. This elution pattern was the same in stem cells and differentiated cells (Figure 3.13). However the signal for MDMX was considerably weaker in differentiated cells (Figure 3.13). In contrast to MDMX, the majority of MDM2 is eluted in fractions that corresponded to a much smaller molecular weight than the fractions where p53 eluted. From the twenty-eight fractions that I obtained from a sucrose gradient, only six

(fraction 12-18) contained larger amounts both of p53 and MDM2. Yet again, there was no discernible difference in the distribution of MDM2 along the sucrose gradient between mESCs and differentiated cells. Like it was the case for MDMX, the signal for MDM2 was weaker in differentiated cells (Figure 3.13), indicating that both inhibitory proteins MDM2 and MDMX are present in greater amounts in stem cells. The presence of a strong signal for the stem cell marker Oct3/4 in the mESCs lysate demonstrates the pluripotency of the mESCs that were used for this experiment (Figure 3.13). The presence of a signal for α7 in fractions 12-18 of the different gradients shows that the elution profile of the different gradients is comparable. (Figure 3.13).

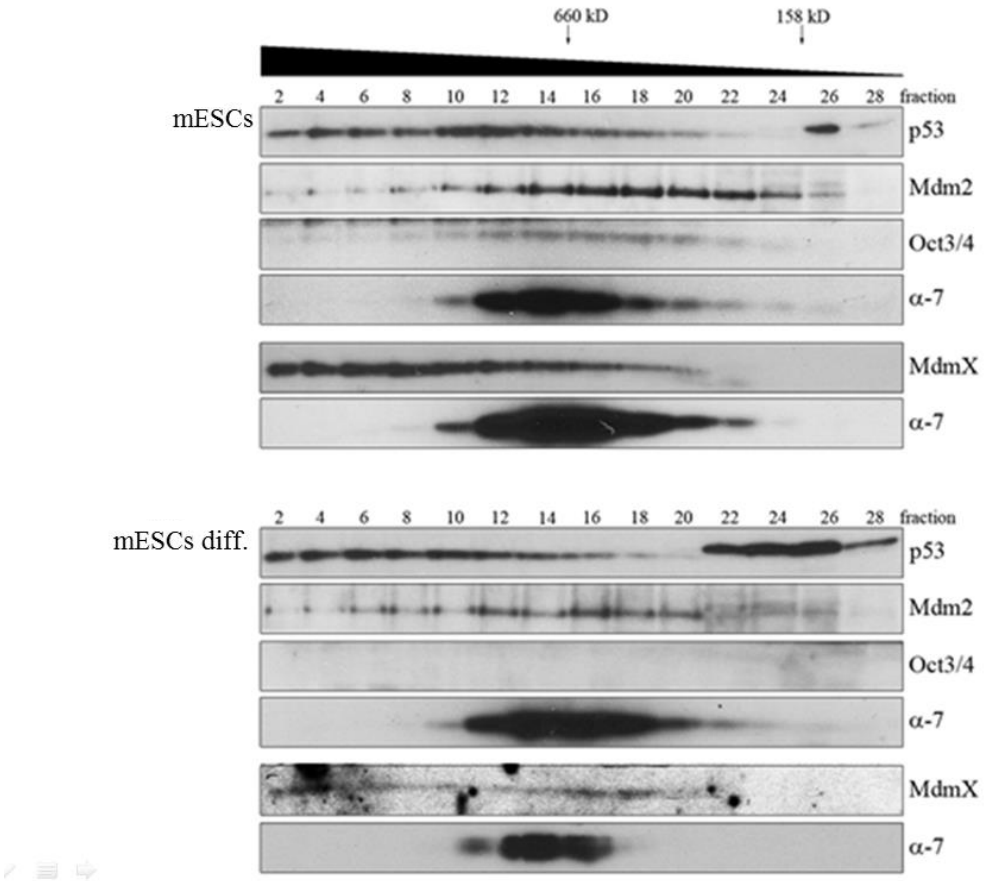


**Figure 3.13 | MDMX co-elutes with p53 from sucrose gradients.** mESCs and mESCs that have been differentiated by incubation with retinoic acid for seven days (mESCs diff.) were lysed in a mild lysis buffer and processed as described in the legend to figure 3.12. The abundance of p53 was detected by hybridization with anti-p53 antibody 1C12 (diluted 1:0,000). Abundance of MDM2 (4B2, diluted 1:1,000), MDMX (diluted 1:3,000), Oct3/4 (diluted 1: 2,000) and of α-7

(MCP72, diluted 1:2,000), for internal control, was monitored by Western blotting.

Since the results from the sucrose gradient centrifugation suggested that the amount of MDM2 and MDMX might be decreased during differentiation, I investigated the abundance of MDM2 and MDMX in stem cells and during differentiation in more detail. I plated mESCs, cultured them in complete DMEM medium supplemented with retinoic acid and harvested cells on day 0, 1, 3 and 5 after retinoic addition. I then monitored abundance of MDM2 and MDMX by western blotting.

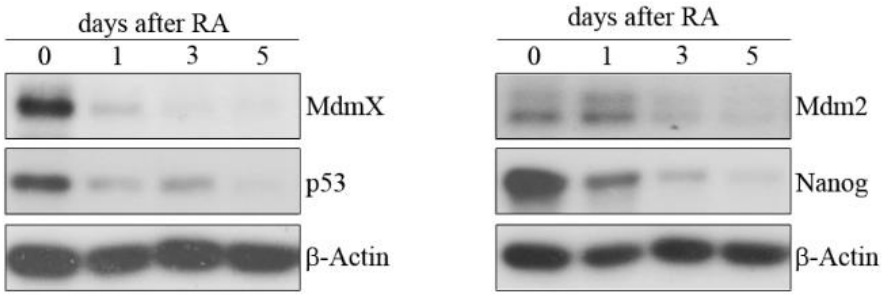


**Figure 3.14 | Abundance of MDM2 and MDMX is decreased during differentiation of mESCs.** mESCs were treated with retinoic acid for differentiation. At the indicated days after addition of retinoic acid, cells were collected and lysed. 30µg of the lysate were separated by SDS-PAGE and the proteins were transferred onto a PVDF membrane. Abundance of MDMX (antibody diluted 1:3,000), p53 (1C12, diluted 1:10,000) and MDM2 (4B2, diluted 1:1,000) was monitored by western blotting as described in the legend to figure 3.1. Abundance of Nanog (C4, diluted 1:2,000) was determined to control for stemness and of β-Actin (I-19, diluted 1:1,000) for loading control.

As shown in figure 3.14, MDMX levels were very high in stem cells and decreased rapidly during differentiation. Already one day after the treatment of stem cells with retinoic acid, the signals for MDMX was strongly decreased, even stronger than that of the stem cell marker Nanog (Figure 3.14). Consistent with previous reports, p53 levels were also decreased during differentiation (Sabapathy et al., 1997; Solozobova and Blattner, 2010). However, while the decrease in the abundance of p53 was slowed down, after an initial strong drop, which allowed a clear detection of p53 at day 3 after the initiation of differentiation, abundance of MDMX was further strongly decreased and was basically undetectable three days after addition of retinoic acid (Figure 3.14). Abundance of MDM2 was also decreased during differentiation, but

this decrease was slower and weaker than that of p53 or MDMX (Figure 3.14).

After having found that MDMX is highly abundant in stem cells and downregulated during differentiation, I wondered whether MDMX indeed regulates p53 activity in mESCs. To test this, I transfected mESCs with a siRNA targeted against MDMX. As the *mdm2* gene is a transcriptional target of p53, I monitored abundance of the MDM2 protein to examine p53 activity (Barak et al., 1993).

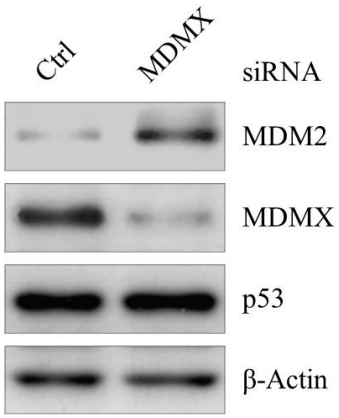


**Figure 3.15 | Downregulation of MDMX increases MDM2 protein levels in mESCs.** mESCs were transfected with a siRNA that was directed against *MDMX* or with a control siRNA. 48h after transfection, cells were lysed. 30µg protein were separated by SDS PAGE and transferred onto a PVDF membrane. Abundance of MDM2 (4B2 diluted 1:1,000), p53 (1C12, 1:1,000) and MDMX (antibody diluted 1:3,000) was performed as described in the legend to figure 3.1. Hybridization with an antibody directed against β-Actin (I-19, diluted 1:1,000) was used to monitor equal loading of the gel.

As shown in figure 3.15, downregulation of MDMX results in a strong increase in the amount of the MDM2 protein, while the amount of the p53 protein remained unchanged (Figure 3.15). This result suggests that MDMX controls p53 activity rather than p53 protein levels. The decrease in the signal for MDMX shows that the downregulation was successful. Similar intensity of the signals for β-Actin show that equal amounts of proteins were loaded onto the gel (Figure 3.16).

Since MDMX expression was decreased during differentiation and MDMX expression seemed to be important for controlling p53 activity in stem cells, I

wondered that by which mechanism MDMX could be responsible for the high amount of MDMX in mESCs. In tumor cells, alternative splicing is an efficient mechanism to modulate MDMX protein levels (Lenos et al., 2012). In collaboration with Jean-Christoph Marine in Gent, Belgium, I investigated whether the splicing of MDMX may differ between stem cells and differentiated cells.

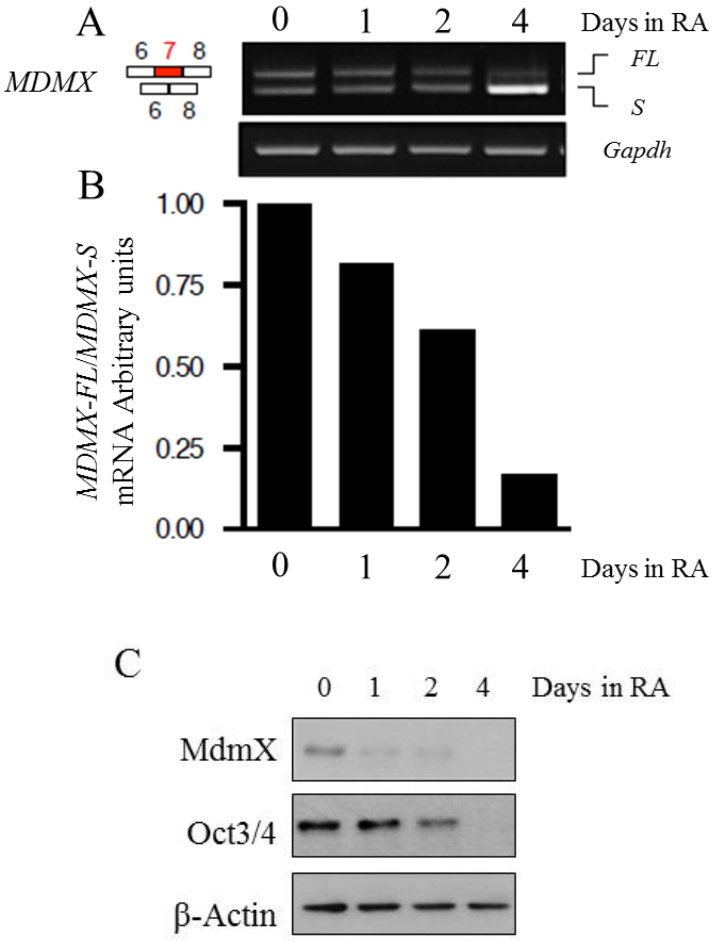


**Figure 3.16 | Inclusion of Mdm4 exon 7 is decreased upon differentiation of mEScs.** mESCs were treated with retinoic acid (RA) for differentiation. At the indicated days, cells were harvested and divided into two aliquots. **A.** RNA was prepared from one of the aliquots and the abundance of the splice products *MDMX-FL* (full length) and *MDMX-S* (short form) was performed by PCR using isoform-specific primers. *Gapdh* levels served for loading control. **B**. The intensity of the bands for the *FL*- and *S*-forms was quantified and the ratio between the two bands was calculated. The ratio between *MDMX-FL* and *MDMX-S* of mESCs was set to 1. **C.** 30µg of lysate were separated by SDS PAGE and the proteins were transferred to a PVDF membrane. Abundance of MDMX (antibody diluted 1:3,000) was determined as described in the legend to figure 3.1. Hybridisation with an antibody directed against Oct3/4 (C10, diluted 1:2000) was performed to monitor differentiation and of β-Actin (I-19, diluted 1:1,000) for loading control.

Therefore, I induced differentiation in mESCs by incubating the cells with retinoic acid. At day 0, 1, 2 and 4 after addition of retinoic acid, I harvested the cells and divided each sample into two aliquots. One of the aliquots I used to monitor MDMX, and Oct3/4 levels as an indication for ongoing differentiation. The second aliquot, I sent to Jean-Christoph Marines' lab where the RNA was isolated and MDMX splicing monitored.

In agreement with the decrease of MDMX protein levels during differentiation, we observed that the amount of the full lengths *MDMX RNA* (*MDMX-FL*) was reduced (Figure 3.16A) while the amount of the shorter form of the *MDMX* RNA (*MDMX-S*) was increased (Figure 3.16A). Since rather the ratio between *MDMX-S* and *MDMX-FL* than the absolute abundance seems to be important for the abundance of the MDMX protein (Jean-Christoph Marine, personal communication), we calculated the ratio between *MDMX-FL* and *MDMX-S*. As shown in figure 3.16B, the ratio of *MDMX-FL and MDMX-S* parallels the decrease in MDMX protein levels in mESCs during differentiation (Figure 3.16B). Part C of the figure shows that Oct3/4 was highly abundant in mESCs. After initiation of differentiation, the intensity of the signal for Oct3/4 declined and was undetectable 4 days after differentiation (Figure 3.16C). In consistency with the result shown in figure 3.14, the MDMX protein level also decreased during differentiation (Figure 3.16C).

## 3.7 Splicing of p53 is altered during retinoic acid induced differentiation

Another possibility for the loss of p53's antiproliferative activity in stem cells could be alternative splicing of p53. For instance, overexpression of the p53-isoform Δ133p53α or loss of the expression of the p53-isoforms p53β and p53γ was found in breast cancer cells (Anensen et al., 2006; Bourdon et al., 2005; Bourdon et al., 2011).

As I already observed changes in the splicing of MDMX during differentiation, I considered it very likely that p53 may also be alternatively spliced during differentiation.

In order to investigate this possibility in more detail, I induced differentiation of stem cells by retinoic acid and harvested the cells at 0, 2, 4 and 6 days after induction of differentiation. To control for the p53-specificity of the antibodies, I also employed p53-negative stem cells that I also induced to differentiate. I lysed the cells and determined the abundance of different p53 isoforms by hybridizing the membrane with the isoform-specific antibody SAPU that was provided to me by Jean-Christoph Bourdon, Dundee, Scotland.

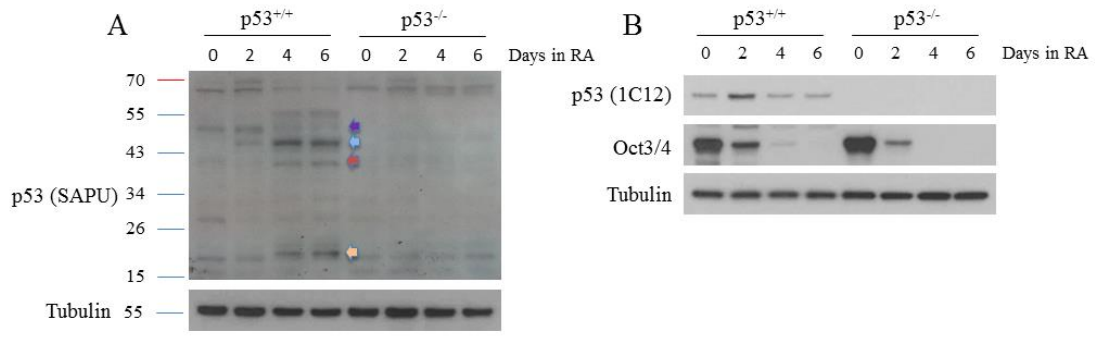


**Figure 3.17 | Abundance of p53 isoforms is altered after retinoic acid induced differentiation of mESCs**. mESCs were treated with retinoic acid (RA) for differentiation. At the indicated days, cells were harvested and divided into two aliquots. **A.** 30µg of the lysate of one of the aliquots were mixed with the 4×stock solution NuPAGE® LDS Sample Buffer, loaded onto a NuPAGE® 10% Bis-Tris polyacrylamide gel and separated in 1×NuPAGE® MOPS SDS Running buffer supplemented with 0.2% Antioxidants. The proteins were transferred onto a PVDF membrane and abundance of p53 was detected by hybridization with the anti-p53 antibody SAPU (diluted 1:2,000) as described in the legend to figure 3.1. Hybridisation with an antibody directed against tubulin (diluted 1:3,000) was used for loading control.Arrows point to bands that are absent in p53-negative cells and whose intensity is changed during differentiation. **B.** The second aliquot was lysed in NP40 lysis buffer. 30µg of the lysate were separated by SDS-PAGE and transferred onto a PVDF membrane. Abundance of p53 was detected by hybridization with the anti-p53 antibody 1C12 (diluted 1:10,000) as described in the legend to figure 3.1. Abundance of Oct3/4 (C10, diluted 1:2000) was determined to control for stemness and hybridisation with an antibody directed against tubulin (diluted 1:3,000) was used for loading control.

When the membranes where hybridized with the anti-p53 antibody SAPU, the p53

signal at around 53kDa was decreased with ongoing differentiation (purple arrow, Figure 3.17A). Concomitantly, a signal around 48kDa and a faint signal of a slightly smaller size were increased, which most likely represent the Δ40p53 and the Δ40p53AS isoforms (blue arrow, Figure 3.17A). In addition, two other signals were increased after retinoic acid induced differentiation, one at around 40kDa (red arrow) and the other one at around 18kDa (bright yellow arrow; Figure 3.19A). Since no isoforms of this size have been described as yet, these bands may represent novel and eventually stem cell-specific isoforms of p53. Most importantly, none of these signals come up in p53-negative stem cells, demonstrating the specificity of the SAPU antibody. Part B shows the abundance of total p53. The decrease in the signal for Oct3/4 reflects ongoing differentiation after addition of retinoic acid to the stem cells.

## 3.8 Wild-type p53 controls a similar set of target genes in stem cells as mutant p53 in differentiated cells.

As described above, the anti-proliferative activity was compromised in mESCs (Figure 3.5). Since there is a high amount of p53 present in stem cells, I wondered whether p53 might have other functions in stem cells than inhibiting proliferation. Such a function could be quite different from its functions in differentiated cells. To address this question, I performed an RNA-sequencing experiment. I extracted RNA from wild-type mESCs and from mESCs with a homozygous deletion of the *p53* gene and handed the RNA over to our sequencing department where the RNA first underwent a quality control test. The RNA samples showed no sign of degradation with more than 89% of the fragments passing the illumine chastity filter. (Table 3.1).

| Sample ID | Description | Yield (Mbases) | % PF | # Reads | % of >= Q30 | Mean Quality Score (PF) |
|---|---|---|---|---|---|---|
| d0_mut | p53-/- | 3.946 | 89,34 | 88.339.574 | 94,66 | 37,24 |
| d0_wt | D3 | 3.257 | 89,67 | 72.652.004 | 94,81 | 37,3 |

**Table 3.1 | Quality of the RNA.** RNA was prepared from mESCs with wild-type p53 (D3) and of mESCs with genetically deleted p53 ($p53^{-/-}$) and tested for quality. Yield: numbers of bases for

each sample; PF: clusters passing illumina chastity filter, (readings with no overlapping or unclear signal) Reads: number of fragment that were read; %of >=Q30: percentage of fragments with misreadings smaller than $2^{-30}$; Mean Quality Score (PF): average score of the fragments with less than $2^{-30}$ misreadings.

The sequencing resulted in more than sixty-seven million reads per sample each of which being 50 nucleotides long with a mean Phred quality score over 35 and a reading errors smaller than two to the minus thirty-five.

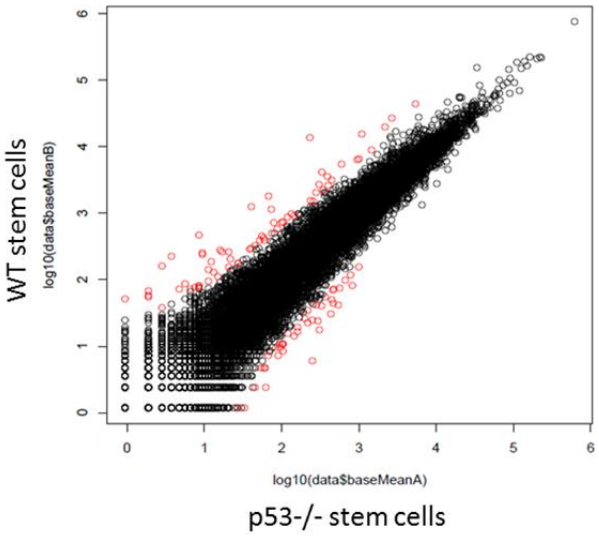


**Figure 3.18 | p53 controls gene expression in mESCs.** The transcriptome of wild-type and p53-negative (*p53*$^{-/-}$) mESCs cells was analysed by RNA sequencing. The graph was generated using the data analyzing software R. The expression of each gene was calculated in $\log_{10}$, and plotted. Genes with a fold change≥2 in their expressions are shown as red circles.

The reads were mapped against the mouse genome M37 database and gene expression was determined by counting for each gene the number of reads that overlapped with the annotation in the Ensembl release 67 database. Differences in gene expression were determined with the R package DESeq.

As shown in figure 3.20, the majority of the expressed genes overlapped between p53-positive and p53-negative mESCs, indicating that expression of most of the genes is independent of p53 (Figure 3.20). However, some genes were differently expressed and either upregulated or downregulated in wild-type mESCs (Figure 3.20, Table 3.2).

Most interestingly, among the genes that are induced by p53 in stem cells are several proto-oncogenes (Table 3.2).

| Gene | $p53^{+/+}/p53^{-/-}$ (mESCs) | | |
|---|---|---|---|
| | ↑ | ↓ | → |
| *c-fos* | × | | |
| *c-myc* | × | | |
| *c-jun* | × | | |
| *mdm2* | × | | |
| *akt1* | × | | |
| *mdmx* | | | × |
| *igf2* | × | | |
| *lef1* | | × | |

**Table 3.2 | Examples of genes regulated by p53 in mESCs.** RNA was prepared from wild-type and $p53^{-/-}$ mESCs, transcribed into cDNA and sequenced. Abundance of transcripts of the individual genes was determined and normalized to an internal control. ↑, gene expression was induced by p53; ↓, gene expression was repressed by p53; →, gene expression was not regulated by p53.

To consolidate the result from RNA sequencing, I performed qRT-PCR for some of the genes. Therefore, I prepared RNA from mESCs and from their p53-negative counterpart, transcribed this RNA into cDNAs and performed SYBR-GREEN based quantitative real time PCR. In accordance with data from differentiated cells (Riley et al., 2008) and from RNA sequencing, expression of *mdm2* and *p21* was higher in p53-positive mESCs (Figure 3.19A). Moreover, and in agreement with the RNA sequencing data (Table 3.2), expression of *akt1*, *c-myc*, *c-jun* and *igf2* was also significantly higher in p53-positive mESCs than in p53-negative mESCs (Figure 3.19B). These increases were not due to an overall increase in gene expression as e.g. expression of *lef1* was reduced in wild-type ESCs compared to $p53^{-/-}$ mESCs (Figure 3.19B).

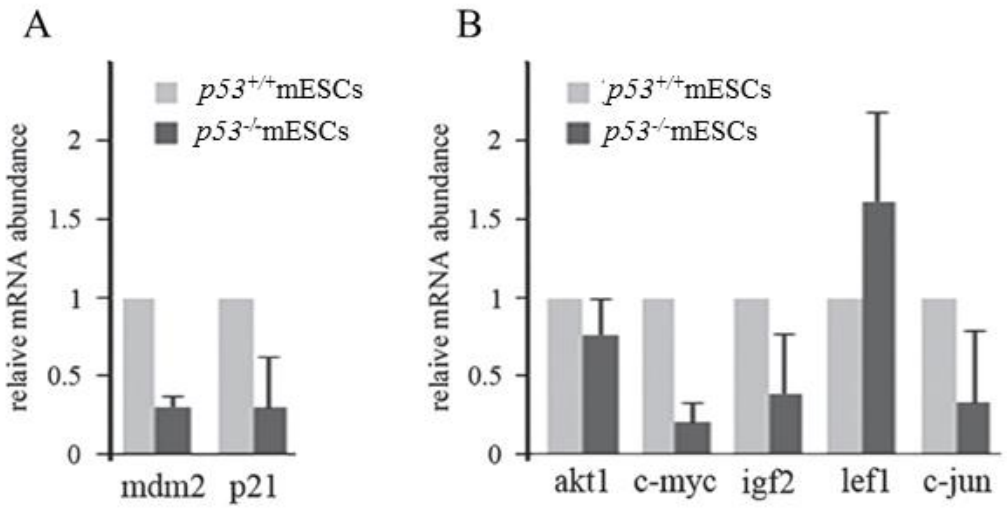


**Figure 3.19 | Several proto-oncogenes are induced by p53 in stem cells.** RNA was prepared from *p53*$^{+/+}$ mESCs and from p53 their negative counterparts (*p53*$^{-/-}$ mESCs) and transcribed into cDNA. The cDNA was diluted in nuclease free water and 4µl of this diluted cDNA solution was used to perform SYBR-GREEN-based quantitative real time PCR using gene-specific primers. For internal control, abundance of the RNA of the housekeeping gene RibPO was determined. The relative abundance of specific cDNAs was calculated with the $\Delta\Delta C_T$ method. Blotted are the mean values and error bars of two independent experiments. Relative abundance of the specific RNA in wild-type mESCs was set to 1. **A.** Classic p53 target genes. **B.** targets of wild-type p53 specifically in mESCs.

To see whether the induction of these stem cell-specific target genes of p53 is also translated into proteins, I performed western blotting. I harvested p53-positive and p53-negative mESCs, lysed the cells in NP-40 lysis buffer and separated the extracts by SDS-PAGE. After transfer I hybridised the membrane with an antibody directed against c-Jun and with antibodies directed against p53, and β-Actin for control.

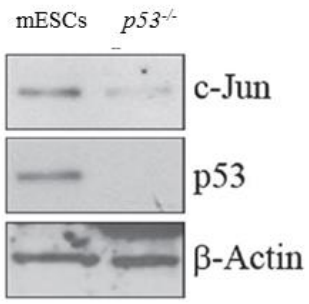


**Figure 3.20 | c-Jun is induced by p53 in mESCs.** Wild-type mESCs and mESCs with a genetic deletion of p53 (*p53*$^{-/-}$) were lysed. 30µg of the lysate were separated by SDS PAGE and

transferred onto a PVDF membrane. Abundance of c-Jun and p53 was monitored by hybridization with an anti-c-Jun (H-79, diluted 1:2,000) and an anti-p53 (1C12, diluted 1:10,000) antibody respectively as described in the legend to figure 3.1. Abundance of β-Actin (I-19, diluted 1:1,000) was determined for loading control.

As shown in figure 3.20, mESCs with wild-type p53 possessed more c-Jun protein than *p53*$^{-/-}$ mESCs (Figure 3.20).

To further support that p53 regulates proto-oncogenes such as *c-jun* and *c-myc* in mESCs, I treated the cells with Nutlin, a chemical activator of Mdm2 that fits into the p53-binding pocket on the Mdm2 protein and thus prevents its interaction with p53, resulting in increased p53 levels and activity (Vassilev et al., 2004). If these proto-oncogenes are indeed induced by p53 in mESCs, expression of these genes should be elevated after Nutlin treatment in p53-positive but not in p53-negative mESCs. To investigate this rationale, I plated p53-positive and p53-negative stem cells. For control, I employed mESCs that had been differentiated by incubation with retinoic acid for seven days since it has been shown that Nutlin stimulates p53 activity in differentiated cells (Vassilev et al., 2004). Half of the cells were treated with Nutlin for 24 hours. I then monitored abundance of c-Jun by Western blotting and abundance of *c-myc* by qRT-PCR. To monitor the activity of Nutlin, I also measured the abundance of MDM2 and *p21* since these classical targets of p53 have been shown to be induced after Nutlin treatment (Giono and Manfredi, 2007)

As shown in figure 3.21, the presence of Nutlin not only resulted in a p53-dependent induction of the classical p53 targets *MDM2* and *p21* but also in the induction of c-Jun and *c-myc* in mESCs (Figure 3.21). This induction only occurred in p53-positive mESCs, demonstrating the p53-dependence. Surprisingly, Nutlin also induced c-Jun in differentiated cells. However, while the induction of c-Jun in stem cells was p53-dependent, the induction of c-Jun by Nutlin also occurred in p53-negative cells (Figure 3.21). In contrast, Nutlin did not induce c-myc in differentiated cells. As expected, p53 and its target MDM2 were clearly induced after Nutlin treatment in mESCs and mESCs differentiated cells, proving the authenticity

of this experiment (Figure 3.21).

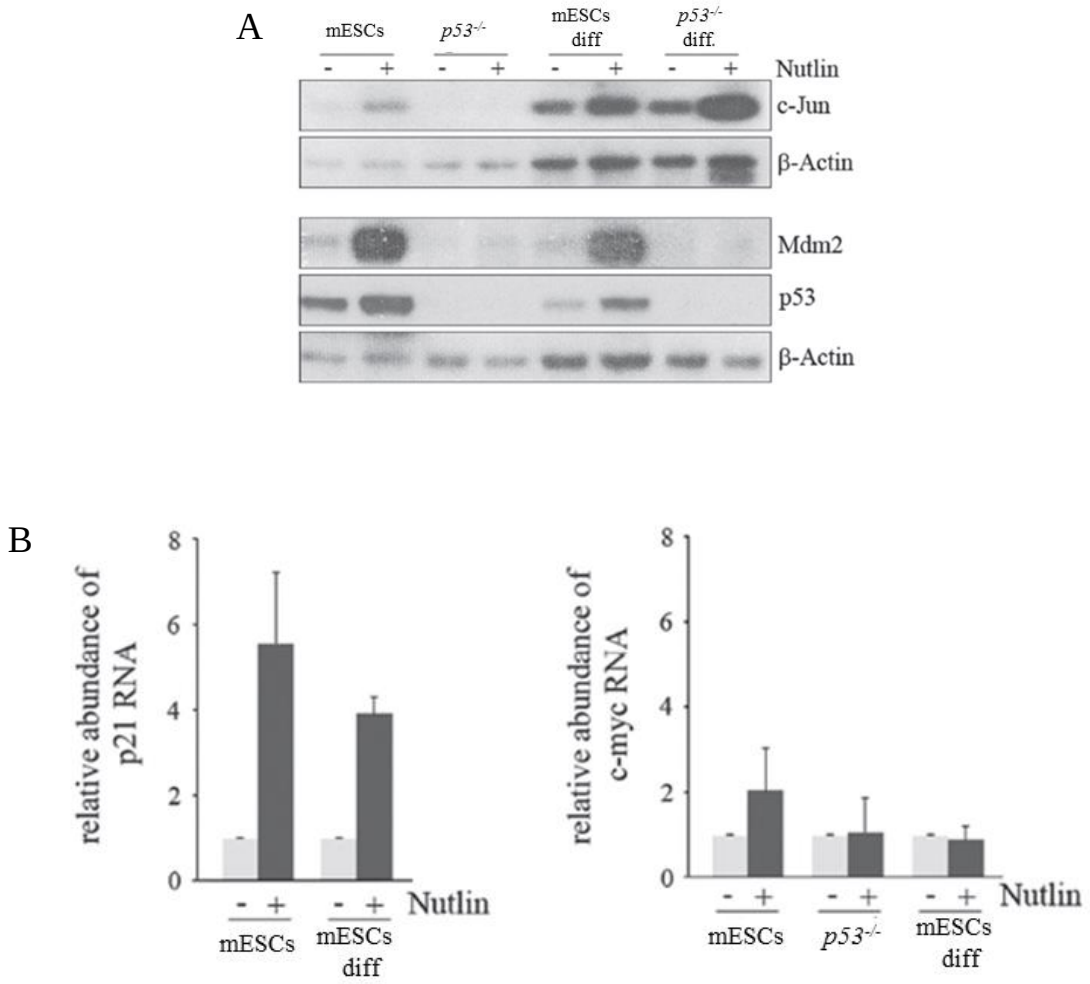


**Figure 3.21 | c-Jun is induced by Nutlin.** mESCs, their p53-negative counterpart ($p53^{-/-}$) and mESCs and $p53^{-/-}$ cells that had been differentiated with retinoic acid for seven days (mESCs diff., $p53^{-/-}$ diff.) were treated with 5 µM Nutlin for 24 hours. **A.** The cells were lysed and 30µg of lysate were separated by SDS PAGE and transferred onto a PVDF membrane. Abundance of c-Jun (H79, diluted 1:2,000), MDM2 (4B2, diluted 1:1,000) and p53 (1C12, diluted 1:10,000) was determined by western blotting as described in the legend to figure 3.1. Hybridization with an antibody directed against β-Actin was performed for loading control. **B.** RNA was prepared and processed as described in the legend to figure 3.19. The graph shows mean values and error bars of two independent experiments. Relative abundance of the specific RNA in mock-treated cells was set to 1.

After having proven that c-Jun and c-myc are induced by the p53 activator Nutlin in mESCs, I reasoned that if p53 indeed induces c-Jun in stem cells, then this induction should be altered when the endogenous regulators of p53 activity, MDM2 and MDMX are downregulated. To follow on this rationale, I transfected mESCs with

siRNAs targeted against p53, MDM2 and MDMX. Seventy-two hours after siRNA transfection I harvested the cells and prepared two aliquots of the cells. One of the aliquots, I lysed and monitored abundance of c-Jun by western blotting. To control for the downregulation, I also monitored p53, MDM2 and MDMX levels by western blotting. I furthermore monitored abundance of the classical p53 targets Bax and Puma by western blotting which are involved in the anti-proliferative activity of p53. From the second aliquot, I prepared RNA and monitored abundance of the p53 target p21 by qRT-PCR.

In support of the previous data, downregulation of p53, or of MDM2 or MDMX, which are endogenous inhibitors of p53, increased the abundance of c-Jun while downregulation of p53, reduced its abundance (Figure 3.22A). In contrast, abundance of Bax or Puma was not changed, indicating that these genes are not controlled by p53 in mESCs under normal growth conditions (Figure 3.22A). Abundance of the p53 target *p21* was reduced after downregulation of p53 but was not grossly altered after downregulation of MDM2 or MDMX (Figure 3.22B).

Since p53 obviously stimulates the expression of some proto-oncogenes in mESC, I wondered whether p53 could be found at the promoter region of these genes. I first checked whether there is a classical p53 binding motif in the promoter and enhancer elements of proto-oncogenes that are induced by p53 in stem cells. However, there was none of the known p53 response elements in any of these genes.

Most interestingly, mutant p53 also stimulates expression of some proto-oncogenes and among the proto-oncogenes that are induced by mutant p53 is c-myc and c-jun, which are also induced by p53 in mESCs (Freed-Pastor and Prives, 2012; Huang et al., 2013; Walerych et al., 2012). I reasoned that if wild-type p53 in mESCs behaves similar to mutant p53 in human tumour cells, and mutant p53 binds to the c-myc promoter, then wild-type p53 in stem cells might also bind to this region and decided to perform chromatin-immunoprecipitations (ChIP). Since it was already described

that mutant human p53 binds to the c-myc promoter (Huang et al., 2013), I selected primers for ChIP for the murine *c-myc* promoter that corresponded to that region. For *akt1* or *c-jun*, I could not find published promoter sequences to which mutant p53 binds. However, it was reported that mutant p53 preferentially and autonomously binds to G/C-rich DNA elements around transcription start sites of several genes (Quante et al., 2012). I therefore checked whether the transcription start sites of akt1 and *c-jun* contain G/C-rich elements. As this was the case, I designed primers that would allow amplifying this region.

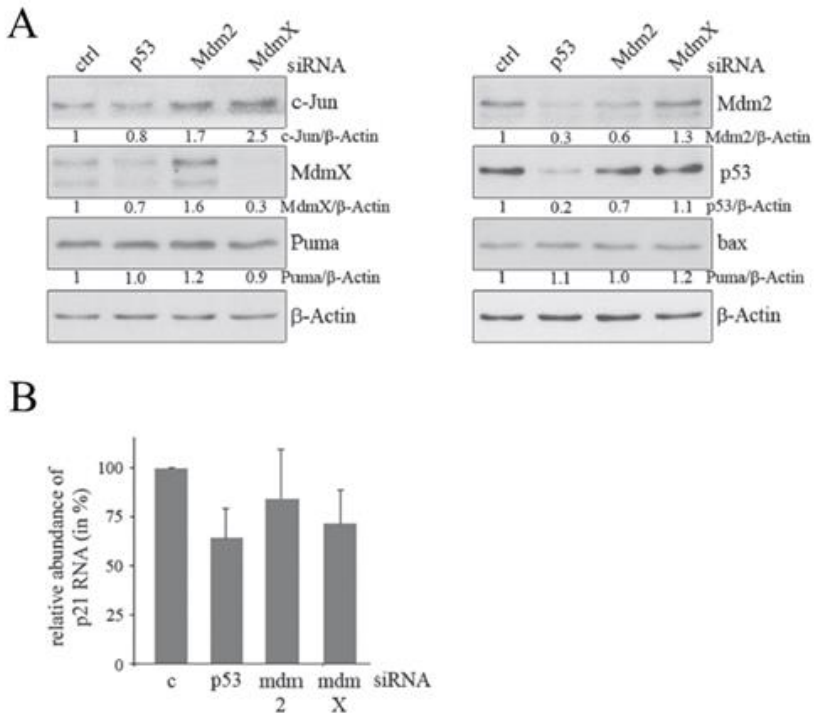


**Figure 3.22 | Downregulation of MDM2 or MDMX increases abundance of c-Jun in mESCs.** mESCs were transfected with a siRNA targeted against p53 (40µM, f.c.), MDMX (40µM, f.c.), MDM2 (40µM, f.c.) or with a control siRNA (40µM, f.c.). After harvesting, the cells were separated into two parts. **A.** One of the samples was lysed with NP-40 lysis buffer and 30 µg of cellular protein were separated by SDS PAGE and transferred onto a PVDF membrane. The abundance of c-Jun (H-79, diluted 1:2,000), Puma (diluted 1:5,000), Bax (diluted 1:5,000), p53 (1C12, diluted 1:10,000), MDM2 (4B2, diluted 1:1,000) and MDMX (diluted diluted 1:3,000) was determined as described in the legend to Figure 3.1. **B.** From the second part, RNA was prepared and processed as described in the legend to figure 3.19. The graph shows mean values and error bars of two independent experiments. Relative abundance of *p21* RNA in control siRNA-transfected cells was set to 100%.

I then plated mESCs, their p53 negative derivatives (*p53*$^{-/-}$) and mESCs and *p53*$^{-/-}$ mESCs that had been differentiated by incubation with retinoic acid for seven days. I fixed the protein/DNA complexes by incubation with formaldehyde, lysed the cells, isolated the nuclei, fragmented the DNA by sonication and precipitated p53 with an polyclonal antibody directed against the tumor suppressor protein. After immunoprecipitating p53, I removed the cross-links between protein and DNA, digested all proteins by incubation with proteinse K, eluted the DNA and performed RT-PCR with the selected primers. To control for the ChIP procedure, I included a PCR reaction with primers corresponding to the p53 binding site in the *MDM2* gene, a classical target of p53 that was also regulated by p53 in mESCs (Table 3.2, Figures 3.19, 3.21, 3.22)

As shown in figure 3.23, the promoter region of *c-jun*, *akt1* and *c-myc* indeed co-precipitated with the p53 protein when an immunoprecipitation was performed with an anti-p53 antibody. The intensity of the signal for the *c-jun*, *akt-1* and *c-myc* promoters was, moreover, comparable to the intensity of the signal for the *mdm2* promoter, a classical and well-known p53 target (Figure 3.23A) (Freedman et al., 1997), showing that p53 is indeed found around the transcriptional start site of these genes. Importantly, I could only precipitate *akt1*, *c-myc* and *c-jun* DNA with an anti-p53 antibody in p53-positive stem cells (Figure 3.23A), but not in p53-negative stem cells. I could also not precipitate these DNAs in differentiated cells with a p53 antibody (Figure 3.23B), indicating that the transcription of *akt1* or *c-myc* by wild-type p53 is specific for stem cells.

p53 is best known for its anti-proliferative activity in response to DNA damage (Lane, 1993). After having demonstrated that p53 indeed induces a novel set of genes in mESCs, I wondered whether these genes are also induced by p53 in response to DNA damage. To see whether this induction of proto-oncogene in response to DNA damage is p53-dependent, I also employed *p53*$^{-/-}$ mESCs. To induce DNA damage, I applied the topoisomerase inhibitor Etoposide (Eto) that induces DNA strand breaks

(Pommier et al., 2010). I then monitored abundance of p53 and its target genes by Western blotting and qRT-PCR.

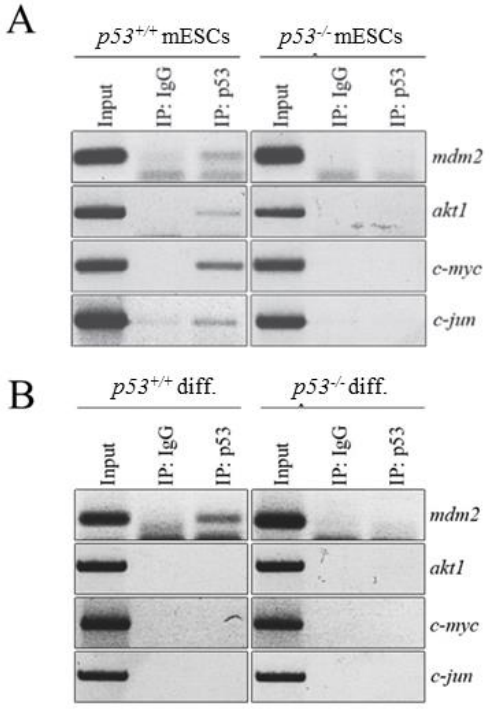


**Figure 3.23 | p53 is associated with the promoter regions of *c-myc*, *c-jun* and *akt1* in stem cells. A.** Wild type mESCs ($p53^{+/+}$ mESCs) and their p53-deficient counterpart ($p53^{-/-}$ mESs) were fixed with 1% formaldehyde (f.c.) for 10 minutes at room temperature. Fixation was stopped byaddition of glycine (0.125M f.c.) and incubation for 5 min at RT. The cells were lysed in 5mM HEPES pH 8, 85mM KCL, 0.5% NP40, 1mM PMSF, 1 µg/ml aproptinin, 1 µg leupeptin, the nuclei were pelleted and suspended in nuclei-lysis buffer (50mM Tris pH 8.1, 10mM EDTA pH 8.0, 1% SDS, 1mM PMSF, 1 µg/ml aproptinin, 1 µg/ml leupeptin). After incubation for 10 min on ice, the lysate was sonicated to achieve an average length of the chromatin of about 400bp. The lysate was divided into three parts. 10% were conserved for input, 45% were incubated with the anti-p53 antibody CM5 and 45% were incubated with IgG O/N at 4 ℃. Protein A agarose was added and the samples were incubated for a further hour. After washing, the cross-links were removed, the proteins digested with proteinase K and the DNA was purified by phenol extraction. The purified DNA was diluted with nuclease free water and 2 µl of the diluted DNA was used to perform PCR. Precipitation with IgG and total cell lysate (Input) were used for positive and negative control. The PCR products were separated by agarose gel electrophoresis and photographed **B.** Differentiated mESCs ($p53^{+/+}$ diff.) and p53-deficient differentiated cells were processed as described in the legend to part A.

As shown previously for the treatment with ionizing irradiation (Solozobova et al., 2009), induction of DNA damage resulted in a strong induction of the p53 protein and

a strong increase in the expression of the classical p53 target genes *mdm2* and *p21* (Figure 3.24). This result shows that p53 in mESCs was activated by the etoposide treatment. When I investigated the expression of c-jun and lef1, which I have found to be induced and downregulated by p53 in mESCs, respectively (Figure Table 3.2), I observed that c-Jun was induced and that lef1 was downregulated after treating mESCs with etoposide. However, c-Jun was also induced and lef1 also downregulated in p53-negative cells (Figure 3.24). This result makes it questionable whether p53 indeed contributes to the DNA-damage-dependent regulation of *c-jun* and *lef-1*. *c-myc*, which was normally induced by p53 in stem cells, was even reduced in response to etoposide treatment. Since this reduction also occurred in p53-negative mESCs, this reduction in c-myc RNA abundance was most likely p53-independent (Figure 3.24).

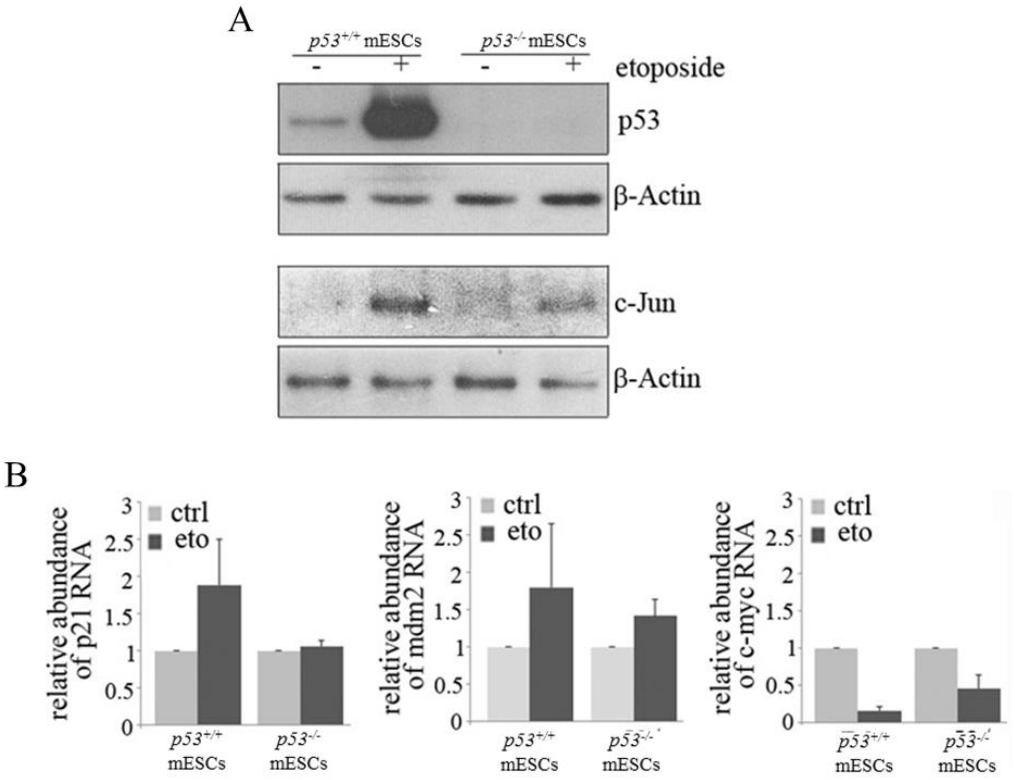


**Figure 3.24 | Involvement of p53 in the regulation of proto-oncogenes in response to DNA damage.** mESCs and their p53-negative counterpart (p53-/-) were treated with 50 µM etoposide or with ethanol for control. 4.5 hours after treatment, cells were harvested. The cells were divided into two aliquots. **A.** One of the aliquots of the cells was lysed, and 30 µg of the lysate were separated by SDS-PAGE and transferred onto a PVDF membrane. Abundance of p53 and c-Jun was monitored by hybridization of the membranes with an anti-p53 antibody (1C12, diluted 1:10,000) and an anti-c-Jun antibody (H79, diluted 1:2,000) as described in the legend to figure

3.1. **B.** From the second aliquot, RNA was prepared and processed as described in the legend to figure 3.19. The graph shows mean values and error bars of two to three independent experiments. Relative abundance of the specific RNA in mock-treated cells was set to 1.

Overall, these results demonstrate that p53 is active in mESCs and capable of influencing the transcriptional program. Most remarkably, p53 controls expression of a different set of genes in mESCs than in differentiated cells. Curiously, several of the genes that are controlled by wild-type p53 in mESCs are controlled by mutant p53 in tumour cells.

# 4 DISCUSSIONS

## 4.1 The p53 protein is primarily localized in the nuclear compartment in mESCs.

In order to explain how stem cells maintain a high proliferation rate regardless of a high amount of p53, I firstly monitored the localization of p53 in mESCs. Since p53 is primarily a transcription factor, the import and retention of p53 in the nucleus is important for its function. Therefore, an abnormal subcellular localization, i.e. in the cytoplasm, could be a reason for its weak effect on cell growth. Indeed previous reports claimed that p53 is localized in the cytoplasm after detecting p53 by immunofluorescence staining and cell fractionation with the anti-p53 antibodies Pab421 and Pab246 (Aladjem et al., 1998; Solozobova et al., 2009; Han et al., 2008). In consistency with these reports, I also got a signal in the cytoplasm when I was using the anti-p53 antibody Pab421. However, with this antibody, I also observed a signal in the cytoplasm of p53 negative mESCs. This result indicates that the signal in the cytoplasm was due to the poor specificity of the anti-p53 antibodies Pab421. Further exploration of p53's localization in mESCs by using the anti-p53 antibodies 1C12 and CM5 showed that the majority of p53 is localized in the nuclear compartment. Since these antibodies gave no signals in p53-negative cells, they are indeed specific for p53. This result was further confirmed by western blotting after cell fractionation. In consistency with the immunofluorescence staining, the signal around fifty-three kDa that was detected by the antibodies Pab246 and Pab421 in the cytoplasm was unspecific. Only the antibodies 1C12 and CM5 showed a good specificity and here, the p53 signal was primarily in the nuclear compartment in mESCs. However, there was also some p53 in the cytoplasm. This cytoplasmic localization of p53 is, however, not specific for stem cells and is also observed in differentiated cells, where cytoplasmic p53 contributes to the induction of apoptosis Moll et al., 2005). To further confirm the nuclear localization of p53, I have treated a

part of the mESCs with the drug leptomycin B (LMB). LMB inhibits CRM1 (chromosome region maintenance 1)-dependent export of proteins from the nucleus into the cytoplasm (Jang et al., 2003; Kudo et al., 1999; Wolff et al., 1997). CRM1 is a member of the importin-beta superfamily of nuclear transport receptors. These receptors recognize proteins that contain a leucine-rich nuclear export sequence (NES) as it is present in p53 Fornerod et al., 1997; Zhang and Xiong, 2001; Ossareh-Nazari et al., 1997). CRM1 has been shown to be required for the export of p53 from the nucleus into the cytoplasm in differentiated cells (Freedman and Levine, 1998; Stommel et al., 1999). Accordingly, p53 accumulated in the nucleus when tumor cells were treated with LMB, where p53 is primarily localized in nucleus (Hietanen et al., 2000). Therefore, p53 should not accumulate in the nucleus after LMB treatment if p53 would be purely localized in the cytoplasm. After LMB treatment, I observed that p53 indeed accumulated in the nucleus, proving that p53 is indeed localized in the nucleus in mESCs. However, I also observed that p53 accumulated in the cytoplasm after LMB treatment. This result suggests that proteins are involved in the degradation of cytoplasmic p53 that need to be exported from the nucleus. Alternatively, LMB could have additional activities, apart from preventing CRM1-mediated nuclear export.

## 4.2 The anti-proliferative activity of p53 is compromised in mESCs.

p53 is a tumor suppressor protein that suppresses cell proliferation primarily by induction of p21/WAF1 (el-Deiry et al., 1993; Tang et al., 1998), an inhibitor of cyclin-dependent kinase 2 that regulates the cell cycle at the G1/S phase (Xiong et al., 1993; Harper et al., 1993). In addition, p53 represses cell proliferation by inducing Gadd45 and 14-3-3-σ, which inhibit CDC2, the cyclin-dependent kinase that is required to enter mitosis (Hermeking et al., 1997; Wang et al., 1999).

Since p53 is obviously localized in the nucleus in mESCs, and also since its abundance is higher in mESCs than in somatic cells, I wondered how stem cells can proliferate fast regardless of the high amount of p53 which normally has an anti-proliferative effect when it is localized in the nucleus. When I investigated the proliferation of p53-positive and p53-negative mESCs, I found that the proliferation rate of p53 positive mESCs is similar to that of p53 negative mESCs. Only on day3 and day4, I observed a minor difference, which was probably due to a reduction in the stemness of the mESCs since they were cultivated four days without sub-culturing them. Yet, mESCs is a sensitive cell type and requires passaging at least every two days to maintain their stem cell properties. This result explains how mESCs can proliferate despite having high amounts of p53, namely by switching off p53's anti-proliferative activity.

## 4.3 p53 is differently modified in stem cells and differentiated cells

Since p53 is nuclear in stem cells as it is in differentiated cells, the localization of p53 cannot be the reason for its inactivation in mESCs. However, p53's activity is largely controlled by posttranslational modifications (PTMs) (Appella and Anderson, 2001; Bode and Dong, 2004; Brooks and Gu, 2003; Oren, 1999; Vogelstein et al., 2000; Vousden and Lu, 2002). I therefore wondered whether distinct PTM in mESCs and differentiated cells could account for p53's compromised activity in stem cells. p53 is regulated by PTMs in two ways: i) PTMs control p53 stability and ii) PTMs alter the activity of preexisting p53 molecules (Boehme and Blattner, 2009). For the regulation of p53 stability, p53 is primarily modified by ubiquitin-ligases. The most well-studied ubiquitin ligase is MDM2, which induces both monoubiquitination and polyubiquitination of p53 Li et al., 2003). Polyubiquitinated p53 is recognized by 26S proteasomes where it is subsequently degraded (Love et al., 2013). Apart from

ubiquitin, p53's stability is also regulated by other small ubiquitin-like proteins, such as SUMO (small ubiquitin-like modifier) (Rodriguez et al., 1999) and NEDD8 (Xirodimas et al., 2004). The activity of p53 is furthermore regulated by phosphorylation, acetylation and methylation (Chehab et al., 2000; Sakaguchi et al., 2000; Shieh et al., 2000). Yet it should be noted that several of these modifications may have a clear function under experimental condition but may not modify the p53 protein under physiologic conditions.

To see whether p53 is differently modified in mESCs and differentiated cells, I employed commercially available antibodies against acetylated p53 at lysine 379, or phosphorylated at serine 18, serine 9 or at serine 389 (corresponding to human lysine 382, serine 6, serine 15 and serine 392 respectively). All these modifications enhance p53's transcriptional activity. Phosphorylation of serine-15 decreases its affinity for its negative regulator MDM2 and promotes its transcriptional activity by recruiting the transcriptional coactivator p300 (Lambert et al., 1998). Phosphorylation of serine 392 is induced in response to UV exposure (Keller and Lu, 2002), and increases the stability of p53's tetramers and inhibits the exportation of p53 into the cytoplasm (Kim et al., 2004; Sakaguchi et al., 1997). Acetylation of p53 at lysine 379, on the other hand, is one of the most important acetylation sites. This acetylation is stimulated by phosphorylation of p53 at the N terminal domain since this phosphorylation recruits p300/CBP that subsequently acetylates lysing 379 (Jenkins et al., 2009; Polley et al., 2008). None of the employed antibodies recognized p53 from undamaged cells, neither from stem cells nor from differentiated cells, apart from the antibody that was directed against serine 392, yet the signal was very weak. After DNA damage, p53 was strongly induced and highly modified at serine 15, serine 392 and at lysine 379. Serine 6 was only phosphorylated in differentiated cells and the signal was very weak.

Since none of the modifications occurs in undamaged stem cells, these modifications cannot explain why the anti-proliferative activity of p53 is compromised in mESCs.

Most interestingly, V. Solozobova in our lab has already found that some p53 with a neutral pI (between 6.4 to 8.2) was exclusively present in mESCs and not in differentiated cells. I wondered whether this fraction of p53 differed in phosphorylation and acetylation from p53 of differentiated cells since these modifications could greatly influence the overall charge of the modified protein resulting in a change in the isoelectric point (Zhang et al., 2009; Zhu et al., 2005). Therefore, I treated the cell lysate of mESCs and of differentiated mESCs with λ-phosphatase to remove the phosphate groups of the proteins including p53, or I treated the cells with the inhibitors of histone acetyltransferases trichostatin A and nicotinamide (Bitterman et al., 2002; Gottlicher et al., 2001) to increase the acetylation level of the proteins including p53, After the treatment, I performed 2-dimensional gel electrophoresis followed by Western blotting. I observed that treatment of the lysate of stem cells with λ-phosphatase shifted a fraction of p53 beyond a pI of 8.6. In samples that had not been treated with the phosphatase, there was no p53 with a pI greater than 8.6 at least not to this extend. This result shows that p53 in stem cells is constitutively phosphorylated. For differentiated cells, I obtained a similar result. Here, the negative charge of p53 was also reduced after phosphatase treatment. However, no p53 with a pI beyond 8.6 was detected in these samples. In fact, p53 from differentiated cells that had been treated with phosphatase resembled untreated p53 from stem cells. In addition, the treatment of TSA/NA strongly increased the negative charge of p53 from stem cells resulting in the complete removal of the fraction of p53 with a neutral pI. In differentiated cells, there was also a tendency to increase the fraction of p53 with a very acidic pI between 4.6 and 5.2. The effect was, however, not as obvious as in stem cells. All these evidences indicate that the neutral pI of p53 is determined by phosphorylation and acetylation. However, it remains to be determined which amino acid/acids is/are differently modified with phosphorylation and acetylation in stem cells and differentiated cells.

## 4.4 MDMX controls p53's activity in mESCs.

Since a fraction of p53 with a neutral pI is exclusively present in mESCs, I wondered whether this may affect the association of p53 with other proteins. Sucrose gradient assays proved that the majority of p53 is always associated with other proteins either in mESCs or in differentiated cells. However, there was no clear difference in the elution of p53 from the sucrose gradient no matter whether it was derived from stem cells or from differentiated cells. I also monitored the elution profile of MDM2 and MDMX, two major negative regulators of p53. MDM2, functions as an E3 ubiquitin ligase, that regulates the abundance and activity of p53 (Haupt et al., 1997; Honda et al., 1997; Itahana et al., 2007; Kulikov et al., 2010). MDMX is a close homolog of MDM2. It controls p53 abundance and inactivates p53 in somatic and cancer cells (Finch et al., 2002; Jackson and Berberich, 2000; Shvarts et al., 1997; Shvarts et al., 1996). However, only a minor fraction of MDM2 co-eluted with p53 from sucrose gradients, indicating that less than half of the p53 protein might be associated with MDM2. MDMX, in contrast, has a similar elution profile as p53, which is consistent with the reported ability of these two proteins to associate with each other (Shvarts et al., 1996). MDMX is moreover highly abundant in stem cells and present in negligible amounts in differentiated cells. It is therefore most likely that MDMX is associated with p53 and controls its activity in stem cells. Co-IP experiments and downregulation assays that have been performed in our research group by V. Solozobova and me confirmed that MDMX is associated with p53 and control p53's anti-proliferative activity while downregulation of MDM2 had a minor effect for p53's activity in mESCs (Yan et al., 2015). This result could explain why stem cells are able to proliferate so fast regardless of the high amount of p53. Nevertheless, although MDMX plays an important role for controlling p53's activity in mESCs, recent studies in our lab and others indicate that MDMX may not be the only factor. TRIM25 for instance, a member of the TRIM super family, inhibits p53's transcriptional activity (Zhang et al., 2015). TRIM25 is also more abundant in stem cells than in differentiated cells (Ping Zhang, personal communication), indicating that it may also

contribute to the control of p53 activity in mESCs. This regulatory network of p53 in mESCs needs to be further investigated in the future.

After I have observed that MDMX expression was decreased during differentiation, I wondered by which mechanism MDMX could be regulated. Alternative splicing is an efficient mechanism to modulate MDMX protein abundance (Boutz et al., under revision). Alternative splicing of MDMX decreases the inclusion of exon 7 of MDMX, and the absence of exon 7 results in a very unstable transcript known as *MDMX-S*. The *MDMX-S* isoform contains a premature termination codon and is targeted for non-sense mediated decay (Rallapalli et al., 1999). The consequence of this exon 7 skipping results in overly active p53. In consequence, mice homozygous for the deletion of *MDMX* exon 7 die in utero, just like *MDMX*-null embryos, due to ectopic p53 activation (Bardot et al., 2014; Parant et al., 2001). I observed that the amount of the full lengths *MDMX RNA* (*MDMX-FL*) was decreased during differentiation while the amount of the shorter form of the *MDMX* RNA (*MDMX-S*) with skipped exon7 was increased. Since rather the ratio between *MDMX-S* and *MDMX-FL* than the absolute abundance seems to be important for the fate of MDMX, we also measured the ratio between *MDMX-FL* and *MDMX-S*. Importantly, the ratio of *MDMX-FL and MDMX-S* parallels the decrease in MDMX protein levels in mESCs when the cells have been exposed to the differentiation promoting agent retinoic acid. Thus, it is most likely that alternative splicing contributes to the downregulation of MDMX during differentiation.

## 4.5 Splicing of p53 is altered during retinoic acid induced differentiation

Since I had already found that the splicing of MDMX is altered during differentiation, I wondering whether the splicing of p53 would also be altered, particularly since a

different splicing pattern alters the activity of p53 (Bourdon et al., 2005). The isoform Δ40p53α, for instance, which is generated by alternative splicing of intron 2 and lacks the first 39 amino acids and thus the major transactivation domain 1 (TAD1), has a dominant-negative effect over full length p53. It inhibits the transcriptional activity of full length p53, and impairs p53-mediated growth suppression (Courtois et al., 2002);. It is thus possible that alternative splicing of p53 could contribute to its partial inactivation in mESCs..

In order to investigate whether p53 is spliced differently in stem cells and differentiated cells, I induced differentiation of stem cells by retinoic acid and monitored abundance of p53 by using an antibody against alternatively spliced isoforms as well as an antibody that recognizes full lengths p53. In consistency with previous work (Sabapathy et al., 1997; Solozobova and Blattner, 2010), abundance of full length p53 decreased during retinoic acid induced differentiation, while a signal around 48kDa was increased, which most likely represents theΔ40p53 and the Δ40p53AS isoforms. Most interestingly, two other signals were increased strongly after retinoic acid induced differentiation, one at around 40kDa and the other one at around 18kDa. The signal at around 18kDa represents most likely the recently described p53 isoform p53Ψ (Senturk et al., 2014). An isoform of p53 with a molecular weight around 40 kDa has not been described as yet and may represent a novel and stem cell specific isoform.

## 4.6 Wild type p53 in mESCs controls the transcriptome in a manner similar to mutant p53 in differentiated cells.

Since the abundance of p53 is higher in stem cells than in differentiated cells (Sabapathy et al., 1997; Solozobova and Blattner, 2010), yet its anti-proliferative activity was compromised, I was particularly interested in finding out whether p53

may have another function in stem cells. By performing RNA sequencing, I found that several genes are expressed differently in mESCs and differentiated cells.

Most interestingly, some of the genes that are expressed at a higher level in p53-positive stem cells including *akt1*, *c-myc*, *c-jun* and *igf2* were reported to be transactivated by mutant p53 in cancer cells (Freed-Pastor and Prives, 2012; Walerych et al., 2012). This implies that wild-type p53 in stem cells may have acquired properties of tumor-derived mutant p53. Interestingly, p53 from stem cells associated with the same region of the *c-myc* promoter that is occupied by mutant p53 in differentiated cells (Huang et al., 2013). Further, p53 was associated with GC-rich regions around the transcriptional start site of *c-jun* and *akt1* in mESCs, a property that has been described for mutant p53 in cancer cells (Quante et al., 2012).

As mESCs express wild type p53, it is paradox that p53 acts like mutant p53 in mESCs. Eventually, p53's conformation is altered by chaperones such as the heat shock proteins, which have been reported to influence the affinity of p53 to the promoter region of its targets. Inhibition of heat shock protein 90 by geldanamysin, for instance, diminishes the binding of p53 to the *p21* promoter sequence in differentiated cells (Walerych et al., 2004).

Which mechanisms are responsible for the altered function of p53 in mESCs merits future investigations.

# 5. REFERENCE

Abraham, J., Kelly, J., Thibault, P., and Benchimol, S. (2000). Post-translational modification of p53 protein in response to ionizing radiation analyzed by mass spectrometry. Journal of molecular biology *295*, 853-864.

Aladjem, M.I., Spike, B.T., Rodewald, L.W., Hope, T.J., Klemm, M., Jaenisch, R., and Wahl, G.M. (1998). ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Current biology : CB *8*, 145-155.

Allton, K., Jain, A.K., Herz, H.M., Tsai, W.W., Jung, S.Y., Qin, J., Bergmann, A., Johnson, R.L., and Barton, M.C. (2009). Trim24 targets endogenous p53 for degradation. Proceedings of the National Academy of Sciences of the United States of America *106*, 11612-11616.

Altman, J., and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. The Journal of comparative neurology *124*, 319-335.

An, W., Kim, J., and Roeder, R.G. (2004). Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. Cell *117*, 735-748.

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome biology *11*, R106.

Anderson, M.E., Woelker, B., Reed, M., Wang, P., and Tegtmeyer, P. (1997). Reciprocal interference between the sequence-specific core and nonspecific C-terminal DNA binding domains of p53: implications for regulation. Molecular and cellular biology *17*, 6255-6264.

Anensen, N., Oyan, A.M., Bourdon, J.C., Kalland, K.H., Bruserud, O., and Gjertsen, B.T. (2006). A distinct p53 protein isoform signature reflects the onset of induction chemotherapy for acute myeloid leukemia. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 3985-3992.

Appella, E., and Anderson, C.W. (2000). Signaling to p53: breaking the posttranslational modification code. Pathologie-biologie *48*, 227-245.

Appella, E., and Anderson, C.W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. European journal of biochemistry / FEBS *268*, 2764-2772.

Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S., and Hohfeld, J. (2005). BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. Molecular biology of the cell *16*, 5891-5900.

Avantaggiati, M.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S., and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175-1184.

Ayed, A., Mulder, F.A., Yi, G.S., Lu, Y., Kay, L.E., and Arrowsmith, C.H. (2001). Latent and active p53 are identical in conformation. Nature structural biology *8*, 756-760.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen,

P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., *et al.* (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science *244*, 217-221.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K., and Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.

Balagurumoorthy, P., Sakamoto, H., Lewis, M.S., Zambrano, N., Clore, G.M., Gronenborn, A.M., Appella, E., and Harrington, R.E. (1995). Four p53 DNA-binding domain peptides bind natural p53-response elements and bend the DNA. Proceedings of the National Academy of Sciences of the United States of America *92*, 8591-8595.

Barak, Y., and Oren, M. (1992). Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest. The EMBO journal *11*, 2115-2121.

Barboza, J.A., Iwakuma, T., Terzian, T., El-Naggar, A.K., and Lozano, G. (2008). Mdm2 and Mdm4 loss regulates distinct p53 activities. Molecular cancer research : MCR *6*, 947-954.

Bardot, B., Bouarich-Bourimi, R., Leemput, J., Lejour, V., Hamon, A., Plancke, L., Jochemsen, A.G., Simeonova, I., Fang, M., and Toledo, F. (2014). Mice engineered for an obligatory Mdm4 exon skipping express higher levels of the Mdm4-S isoform but exhibit increased p53 activity. Oncogene.

Bargonetti, J., Manfredi, J.J., Chen, X., Marshak, D.R., and Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes & development *7*, 2565-2574.

Barlev, N.A., Liu, L., Chehab, N.H., Mansfield, K., Harris, K.G., Halazonetis, T.D., and Berger, S.L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Molecular cell *8*, 1243-1254.

Becker, K.A., Ghule, P.N., Therrien, J.A., Lian, J.B., Stein, J.L., van Wijnen, A.J., and Stein, G.S. (2006). Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. Journal of cellular physiology *209*, 883-893.

Bezzi, M., Teo, S.X., Muller, J., Mok, W.C., Sahu, S.K., Vardy, L.A., Bonday, Z.Q., and Guccione, E. (2013). Regulation of constitutive and alternative splicing by PRMT5 reveals a role for Mdm4 pre-mRNA in sensing defects in the spliceosomal machinery. Genes & development *27*, 1903-1916.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. The Journal of biological chemistry *277*, 45099-45107.

Bochar, D.A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W.S., Wang, W., Kashanchi, F., and Shiekhattar, R. (2000). BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. Cell *102*, 257-265.

Bode, A.M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nature reviews Cancer *4*, 793-805.

Boehme, K.A., and Blattner, C. (2009). Regulation of p53--insights into a complex process. Critical reviews in biochemistry and molecular biology *44*, 367-392.

Boesten, L.S., Zadelaar, S.M., De Clercq, S., Francoz, S., van Nieuwkoop, A., Biessen, E.A., Hofmann, F., Feil, S., Feil, R., Jochemsen, A.G., *et al.* (2006). Mdm2, but not Mdm4, protects terminally differentiated smooth muscle cells from p53-mediated caspase-3-independent cell death. Cell death and differentiation *13*, 2089-2098.

Bourdon, J.C. (2007). p53 and its isoforms in cancer. British journal of cancer *97*, 277-282.

Bourdon, J.C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D.P., Saville, M.K., and Lane, D.P. (2005). p53 isoforms can regulate p53 transcriptional activity. Genes & development *19*, 2122-2137.

Bourdon, J.C., Khoury, M.P., Diot, A., Baker, L., Fernandes, K., Aoubala, M., Quinlan, P., Purdie, C.A., Jordan, L.B., Prats, A.C., *et al.* (2011). p53 mutant breast cancer patients expressing p53gamma have as good a prognosis as wild-type p53 breast cancer patients. Breast cancer research : BCR *13*, R7.

Boutz, P.L., Bhutkar, A., and Sharp, P.A. (2015). Detained introns are a novel, widespread class of post-transcriptionally spliced introns. Genes & development *29*, 63-80.

Bouvard, V., Zaitchouk, T., Vacher, M., Duthu, A., Canivet, M., Choisy-Rossi, C., Nieruchalski, M., and May, E. (2000). Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice. Oncogene *19*, 649-660.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., *et al.* (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947-956.

Braastad, C.D., Han, Z., and Hendrickson, E.A. (2003). Constitutive DNase I hypersensitivity of p53-regulated promoters. The Journal of biological chemistry *278*, 8261-8268.

Brooks, C.L., and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Current opinion in cell biology *15*, 164-171.

Bullock, A.N., Henckel, J., and Fersht, A.R. (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. Oncogene *19*, 1245-1256.

Burns, T.F., and El-Deiry, W.S. (2003). Microarray analysis of p53 target gene expression patterns in the spleen and thymus in response to ionizing radiation. Cancer biology & therapy *2*, 431-443.

Caelles, C., Helmberg, A., and Karin, M. (1994). p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature *370*, 220-223.

Cahilly-Snyder, L., Yang-Feng, T., Francke, U., and George, D.L. (1987). Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somatic cell and molecular genetics *13*, 235-244.

Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J., *et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell *116*, 499-509.

Cervantes, R.B., Stringer, J.R., Shao, C., Tischfield, J.A., and Stambrook, P.J. (2002). Embryonic stem cells and somatic cells differ in mutation frequency and type. Proceedings of the National Academy of Sciences of the United States of America *99*, 3586-3590.

Chalkley, G.E., Knowles, P.P., Whitehead, P.C., and Coffer, A.I. (1994). Biochemical characterisation of purified human wild-type p53 overexpressed in insect cells. European journal of biochemistry / FEBS *221*, 167-175.

Chang, C.J., Chao, C.H., Xia, W., Yang, J.Y., Xiong, Y., Li, C.W., Yu, W.H., Rehman, S.K., Hsu, J.L., Lee, H.H., *et al.* (2011). p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nature cell biology *13*, 317-323.

Chehab, N.H., Malikzay, A., Appel, M., and Halazonetis, T.D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes & development *14*, 278-288.

Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell *121*, 1071-1083.

Chen, J., Marechal, V., and Levine, A.J. (1993). Mapping of the p53 and mdm-2 interaction domains. Molecular and cellular biology *13*, 4107-4114.

Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochemical and biophysical research communications *279*, 383-390.

Chipuk, J.E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D.D., and Green, D.R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science *309*, 1732-1735.

Chipuk, J.E., Maurer, U., Green, D.R., and Schuler, M. (2003). Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer cell *4*, 371-381.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-355.

Chuikov, S., Kurash, J.K., Wilson, J.R., Xiao, B., Justin, N., Ivanov, G.S., McKinney, K., Tempst, P., Prives, C., Gamblin, S.J., *et al.* (2004). Regulation of p53 activity through lysine methylation. Nature *432*, 353-360.

Courtois, S., Verhaegh, G., North, S., Luciani, M.G., Lassus, P., Hibner, U., Oren, M., and Hainaut, P. (2002). DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. Oncogene *21*, 6722-6728.

Dai, C., and Gu, W. (2010). p53 post-translational modification: deregulated in tumorigenesis. Trends in molecular medicine *16*, 528-536.

Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., *et al.* (2004). Amplification of Mdmx (or Mdm4)

directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. Molecular and cellular biology *24*, 5835-5843.

Davison, T.S., Yin, P., Nie, E., Kay, C., and Arrowsmith, C.H. (1998). Characterization of the oligomerization defects of two p53 mutants found in families with Li-Fraumeni and Li-Fraumeni-like syndrome. Oncogene *17*, 651-656.

de Waard, H., de Wit, J., Gorgels, T.G., van den Aardweg, G., Andressoo, J.O., Vermeij, M., van Steeg, H., Hoeijmakers, J.H., and van der Horst, G.T. (2003). Cell type-specific hypersensitivity to oxidative damage in CSB and XPA mice. DNA repair *2*, 13-25.

Delavaine, L., and La Thangue, N.B. (1999). Control of E2F activity by p21Waf1/Cip1. Oncogene *18*, 5381-5392.

Demonacos, C., Krstic-Demonacos, M., and La Thangue, N.B. (2001). A TPR motif cofactor contributes to p300 activity in the p53 response. Molecular cell *8*, 71-84.

Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994). A 26 S protease subunit that binds ubiquitin conjugates. The Journal of biological chemistry *269*, 7059-7061.

Deyoung, M.P., and Ellisen, L.W. (2007). p63 and p73 in human cancer: defining the network. Oncogene *26*, 5169-5183.

Di Como, C.J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Molecular and cellular biology *19*, 1438-1449.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B., *et al.* (1990). p53 functions as a cell cycle control protein in osteosarcomas. Molecular and cellular biology *10*, 5772-5781.

Dippold, W.G., Jay, G., DeLeo, A.B., Khoury, G., and Old, L.J. (1981). p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. Proceedings of the National Academy of Sciences of the United States of America *78*, 1695-1699.

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C., and Levine, A.J. (1993). Gain of function mutations in p53. Nature genetics *4*, 42-46.

Dolezalova, D., Mraz, M., Barta, T., Plevova, K., Vinarsky, V., Holubcova, Z., Jaros, J., Dvorak, P., Pospisilova, S., and Hampl, A. (2012). MicroRNAs regulate p21(Waf1/Cip1) protein expression and the DNA damage response in human embryonic stem cells. Stem cells *30*, 1362-1372.

Donovan, P.J., and Gearhart, J. (2001). The end of the beginning for pluripotent stem cells. Nature *414*, 92-97.

Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G.D., Dowd, P., O'Rourke, K., Koeppen, H., and Dixit, V.M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature *429*, 86-92.

Dulbecco, R., Allen, W.R., Bologna, M., and Bowman, M. (1986). Marker evolution during the development of the rat mammary gland: stem cells identified by markers and the role of

myoepithelial cells. Cancer research *46*, 2449-2456.

Dumaz, N., Milne, D.M., and Meek, D.W. (1999). Protein kinase CK1 is a p53-threonine 18 kinase which requires prior phosphorylation of serine 15. FEBS letters *463*, 312-316.

Dumont, P., Leu, J.I., Della Pietra, A.C., 3rd, George, D.L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nature genetics *33*, 357-365.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Espinosa, J.M., and Emerson, B.M. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Molecular cell *8*, 57-69.

Esser, C., Scheffner, M., and Hohfeld, J. (2005). The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. The Journal of biological chemistry *280*, 27443-27448.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.

Fakharzadeh, S.S., Trusko, S.P., and George, D.L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. The EMBO journal *10*, 1565-1569.

Ferguson, J.E., 3rd, Kelley, R.W., and Patterson, C. (2005). Mechanisms of endothelial differentiation in embryonic vasculogenesis. Arteriosclerosis, thrombosis, and vascular biology *25*, 2246-2254.

Fernandez-Fernandez, M.R., Veprintsev, D.B., and Fersht, A.R. (2005). Proteins of the S100 family regulate the oligomerization of p53 tumor suppressor. Proceedings of the National Academy of Sciences of the United States of America *102*, 4735-4740.

Fields, S., and Jang, S.K. (1990). Presence of a potent transcription activating sequence in the p53 protein. Science *249*, 1046-1049.

Finch, R.A., Donoviel, D.B., Potter, D., Shi, M., Fan, A., Freed, D.D., Wang, C.Y., Zambrowicz, B.P., Ramirez-Solis, R., Sands, A.T., *et al.* (2002). mdmx is a negative regulator of p53 activity in vivo. Cancer research *62*, 3221-3225.

Finlan, L., and Hupp, T.R. (2004). The N-terminal interferon-binding domain (IBiD) homology domain of p300 binds to peptides with homology to the p53 transactivation domain. The Journal of biological chemistry *279*, 49395-49405.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083-1093.

Fiscella, M., Ullrich, S.J., Zambrano, N., Shields, M.T., Lin, D., Lees-Miller, S.P., Anderson, C.W., Mercer, W.E., and Appella, E. (1993). Mutation of the serine 15 phosphorylation site of

human p53 reduces the ability of p53 to inhibit cell cycle progression. Oncogene *8*, 1519-1528.

Flaman, J.M., Waridel, F., Estreicher, A., Vannier, A., Limacher, J.M., Gilbert, D., Iggo, R., and Frebourg, T. (1996). The human tumour suppressor gene p53 is alternatively spliced in normal cells. Oncogene *12*, 813-818.

Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer cell *7*, 363-373.

Freed-Pastor, W.A., and Prives, C. (2012). Mutant p53: one name, many proteins. Genes & development *26*, 1268-1286.

Freedman, D.A., and Levine, A.J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Molecular and cellular biology *18*, 7288-7293.

Frescas, D., and Pagano, M. (2008). Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nature reviews Cancer *8*, 438-449.

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Molecular and cellular biology *21*, 1874-1887.

Garritano, S., Gemignani, F., Palmero, E.I., Olivier, M., Martel-Planche, G., Le Calvez-Kelm, F., Brugieres, L., Vargas, F.R., Brentani, R.R., Ashton-Prolla, P., *et al.* (2010). Detailed haplotype analysis at the TP53 locus in p.R337H mutation carriers in the population of Southern Brazil: evidence for a founder effect. Human mutation *31*, 143-150.

Geyer, R.K., Yu, Z.K., and Maki, C.G. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. Nature cell biology *2*, 569-573.

Ghins, E., Colson-van Schoor, M., and Marechal, G. (1984). The origin of muscle stem cells in rat triceps surae regenerating after mincing. Journal of muscle research and cell motility *5*, 711-722.

Ghosh, A., Stewart, D., and Matlashewski, G. (2004). Regulation of human p53 activity and cell localization by alternative splicing. Molecular and cellular biology *24*, 7987-7997.

Gohler, T., Jager, S., Warnecke, G., Yasuda, H., Kim, E., and Deppert, W. (2005). Mutant p53 proteins bind DNA in a DNA structure-selective mode. Nucleic acids research *33*, 1087-1100.

Gorlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science *271*, 1513-1518.

Gottlicher, M., Minucci, S., Zhu, P., Kramer, O.H., Schimpf, A., Giavara, S., Sleeman, J.P., Lo Coco, F., Nervi, C., Pelicci, P.G., *et al.* (2001). Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. The EMBO journal *20*, 6969-6978.

Graunke, D.M., Fornace, A.J., Jr., and Pieper, R.O. (1999). Presetting of chromatin structure and

transcription factor binding poise the human GADD45 gene for rapid transcriptional up-regulation. Nucleic acids research *27*, 3881-3890.

Groll, M., and Huber, R. (2003). Substrate access and processing by the 20S proteasome core particle. The international journal of biochemistry & cell biology *35*, 606-616.

Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A.G., Parant, J., Lozano, G., and Yuan, Z.M. (2002). Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. The Journal of biological chemistry *277*, 19251-19254.

Gu, W., Malik, S., Ito, M., Yuan, C.X., Fondell, J.D., Zhang, X., Martinez, E., Qin, J., and Roeder, R.G. (1999). A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Molecular cell *3*, 97-108.

Gu, W., and Roeder, R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Halazonetis, T.D., and Kandil, A.N. (1993). Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. The EMBO journal *12*, 5057-5064.

Han, M.K., Song, E.K., Guo, Y., Ou, X., Mantel, C., and Broxmeyer, H.E. (2008). SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. Cell stem cell *2*, 241-251.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Haupt, Y., Rowan, S., Shaulian, E., Vousden, K.H., and Oren, M. (1995). Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes & development *9*, 2170-2183.

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Molecular cell *1*, 3-11.

Hietanen, S., Lain, S., Krausz, E., Blattner, C., and Lane, D.P. (2000). Activation of p53 in cervical carcinoma cells by small molecules. Proceedings of the National Academy of Sciences of the United States of America *97*, 8501-8506.

Higashimoto, Y., Saito, S., Tong, X.H., Hong, A., Sakaguchi, K., Appella, E., and Anderson, C.W. (2000). Human p53 is phosphorylated on serines 6 and 9 in response to DNA damage-inducing agents. The Journal of biological chemistry *275*, 23199-23203.

Ho, J.S., Ma, W., Mao, D.Y., and Benchimol, S. (2005). p53-Dependent transcriptional repression of c-myc is required for G1 cell cycle arrest. Molecular and cellular biology *25*, 7423-7431.

Holaska, J.M., and Paschal, B.M. (1998). A cytosolic activity distinct from crm1 mediates nuclear

export of protein kinase inhibitor in permeabilized cells. Proceedings of the National Academy of Sciences of the United States of America *95*, 14739-14744.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science *253*, 49-53.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS letters *420*, 25-27.

Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature *460*, 1132-1135.

Hsu, C.H., Chang, M.D., Tai, K.Y., Yang, Y.T., Wang, P.S., Chen, C.J., Wang, Y.H., Lee, S.C., Wu, C.W., and Juan, L.J. (2004). HCMV IE2-mediated inhibition of HAT activity downregulates p53 function. The EMBO journal *23*, 2269-2280.

Huang, X., Zhang, Y., Tang, Y., Butler, N., Kim, J., Guessous, F., Schiff, D., Mandell, J., and Abounader, R. (2013). A novel PTEN/mutant p53/c-Myc/Bcl-XL axis mediates context-dependent oncogenic effects of PTEN with implications for cancer prognosis and therapy. Neoplasia *15*, 952-965.

Hupp, T.R., and Lane, D.P. (1994). Allosteric activation of latent p53 tetramers. Current biology : CB *4*, 865-875.

Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., Dobbelstein, M., Del Sal, G., Piaggio, G., and Mantovani, R. (2005). Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. Molecular and cellular biology *25*, 3737-3751.

Itahana, K., Mao, H., Jin, A., Itahana, Y., Clegg, H.V., Lindstrom, M.S., Bhat, K.P., Godfrey, V.L., Evan, G.I., and Zhang, Y. (2007). Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. Cancer cell *12*, 355-366.

Jabbur, J.R., Tabor, A.D., Cheng, X., Wang, H., Uesugi, M., Lozano, G., and Zhang, W. (2002). Mdm-2 binding and TAF(II)31 recruitment is regulated by hydrogen bond disruption between the p53 residues Thr18 and Asp21. Oncogene *21*, 7100-7113.

Jackson, M.W., and Berberich, S.J. (2000). MdmX protects p53 from Mdm2-mediated degradation. Molecular and cellular biology *20*, 1001-1007.

Jang, B.C., Munoz-Najar, U., Paik, J.H., Claffey, K., Yoshida, M., and Hla, T. (2003). Leptomycin B, an inhibitor of the nuclear export receptor CRM1, inhibits COX-2 expression. The Journal of biological chemistry *278*, 2773-2776.

Jang, J., Yoo, J.E., Lee, J.A., Lee, D.R., Kim, J.Y., Huh, Y.J., Kim, D.S., Park, C.Y., Hwang, D.Y., Kim, H.S., *et al.* (2012). Disease-specific induced pluripotent stem cells: a platform for human disease modeling and drug discovery. Experimental & molecular medicine *44*, 202-213.

Jenkins, L.M., Yamaguchi, H., Hayashi, R., Cherry, S., Tropea, J.E., Miller, M., Wlodawer, A., Appella, E., and Mazur, S.J. (2009). Two distinct motifs within the p53 transactivation domain bind to the Taz2 domain of p300 and are differentially affected by phosphorylation. Biochemistry *48*, 1244-1255.

Jensen, U.B., Lowell, S., and Watt, F.M. (1999). The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development *126*, 2409-2418.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Joo, H.M., Kim, J.Y., Jeong, J.B., Seong, K.M., Nam, S.Y., Yang, K.H., Kim, C.S., Kim, H.S., Jeong, M., An, S., *et al.* (2011). Ret finger protein 2 enhances ionizing radiation-induced apoptosis via degradation of AKT and MDM2. European journal of cell biology *90*, 420-431.

Jung, H., Seong, H.A., and Ha, H. (2008). Critical role of cysteine residue 81 of macrophage migration inhibitory factor (MIF) in MIF-induced inhibition of p53 activity. The Journal of biological chemistry *283*, 20383-20396.

Kaeser, M.D., and Iggo, R.D. (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proceedings of the National Academy of Sciences of the United States of America *99*, 95-100.

Kakudo, Y., Shibata, H., Otsuka, K., Kato, S., and Ishioka, C. (2005). Lack of correlation between p53-dependent transcriptional activity and the ability to induce apoptosis among 179 mutant p53s. Cancer research *65*, 2108-2114.

Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499-509.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kawamura, T., Suzuki, J., Wang, Y.V., Menendez, S., Morera, L.B., Raya, A., Wahl, G.M., and Izpisua Belmonte, J.C. (2009). Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature *460*, 1140-1144.

Keller, D.M., and Lu, H. (2002). p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2.hSPT16.SSRP1 complex. The Journal of biological chemistry *277*, 50206-50213.

Kern, S.E., Kinzler, K.W., Baker, S.J., Nigro, J.M., Rotter, V., Levine, A.J., Friedman, P., Prives, C., and Vogelstein, B. (1991a). Mutant p53 proteins bind DNA abnormally in vitro. Oncogene *6*, 131-136.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991b). Identification of p53 as a sequence-specific DNA-binding protein. Science *252*, 1708-1711.

Kim, E., and Deppert, W. (2004). Transcriptional activities of mutant p53: when mutations are

more than a loss. Journal of cellular biochemistry *93*, 878-886.

Kim, M.M., Wiederschain, D., Kennedy, D., Hansen, E., and Yuan, Z.M. (2007). Modulation of p53 and MDM2 activity by novel interaction with Ras-GAP binding proteins (G3BP). Oncogene *26*, 4209-4215.

Kim, Y.Y., Park, B.J., Kim, D.J., Kim, W.H., Kim, S., Oh, K.S., Lim, J.Y., Kim, J., Park, C., and Park, S.I. (2004). Modification of serine 392 is a critical event in the regulation of p53 nuclear export and stability. FEBS letters *572*, 92-98.

Ko, L.J., and Prives, C. (1996). p53: puzzle and paradigm. Genes & development *10*, 1054-1072.

Koepp, D.M., and Silver, P.A. (1996). A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? Cell *87*, 1-4.

Kojima, K., Konopleva, M., McQueen, T., O'Brien, S., Plunkett, W., and Andreeff, M. (2006). Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. Blood *108*, 993-1000.

Kress, M., May, E., Cassingena, R., and May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. Journal of virology *31*, 472-483.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiological reviews *87*, 99-163.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proceedings of the National Academy of Sciences of the United States of America *96*, 9112-9117.

Kulikov, R., Letienne, J., Kaur, M., Grossman, S.R., Arts, J., and Blattner, C. (2010). Mdm2 facilitates the association of p53 with the proteasome. Proceedings of the National Academy of Sciences of the United States of America *107*, 10038-10043.

Lam, Y.A., Lawson, T.G., Velayutham, M., Zweier, J.L., and Pickart, C.M. (2002). A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature *416*, 763-767.

Lambert, P.F., Kashanchi, F., Radonovich, M.F., Shiekhattar, R., and Brady, J.N. (1998). Phosphorylation of p53 serine 15 increases interaction with CBP. The Journal of biological chemistry *273*, 33048-33053.

Lane, D., and Gannon, J. (1983). Cellular proteins involved in SV40 transformation. Cell Biology International Reports *7*, 513-514.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Lane, D.P. (1993). Cancer. A death in the life of p53. Nature *362*, 786-787.

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

Lanyi, A., Deb, D., Seymour, R.C., Ludes-Meyers, J.H., Subler, M.A., and Deb, S. (1998). 'Gain of function' phenotype of tumor-derived mutant p53 requires the oligomerization/nonsequence-specific nucleic acid-binding domain. Oncogene *16*, 3169-3176.

Lee, D., Kim, J.W., Seo, T., Hwang, S.G., Choi, E.J., and Choe, J. (2002). SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. The Journal of biological chemistry *277*, 22330-22337.

Lee, K.C., Crowe, A.J., and Barton, M.C. (1999). p53-mediated repression of alpha-fetoprotein gene expression by specific DNA binding. Molecular and cellular biology *19*, 1279-1288.

Lee, M.K., Hande, M.P., and Sabapathy, K. (2005). Ectopic mTERT expression in mouse embryonic stem cells does not affect differentiation but confers resistance to differentiation- and stress-induced p53-dependent apoptosis. Journal of cell science *118*, 819-829.

Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell *112*, 779-791.

Letai, A.G. (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nature reviews Cancer *8*, 121-132.

Leu, J.I., Dumont, P., Hafey, M., Murphy, M.E., and George, D.L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nature cell biology *6*, 443-450.

Leung, K.M., Po, L.S., Tsang, F.C., Siu, W.Y., Lau, A., Ho, H.T., and Poon, R.Y. (2002). The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. Cancer research *62*, 4890-4893.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Levine, A.J., Hu, W., and Feng, Z. (2006). The P53 pathway: what questions remain to be explored? Cell death and differentiation *13*, 1027-1036.

Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M.A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature *460*, 1136-1139.

Li, M., and Huang, J. (2010). A new puzzling role of p53 in mouse embryonic stem cells. Cell cycle *9*, 1669-1670.

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., Baer, R., and Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell *149*, 1269-1283.

Lill, N.L., Grossman, S.R., Ginsberg, D., DeCaprio, J., and Livingston, D.M. (1997). Binding and modulation of p53 by p300/CBP coactivators. Nature *387*, 823-827.

Lin, S., and Riggs, A.D. (1975). The general affinity of lac repressor for E. coli DNA: implications

for gene regulation in procaryotes and eucaryotes. Cell *4*, 107-111.

Lin, T., Chao, C., Saito, S., Mazur, S.J., Murphy, M.E., Appella, E., and Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nature cell biology *7*, 165-171.

Linares, L.K., Hengstermann, A., Ciechanover, A., Muller, S., and Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proceedings of the National Academy of Sciences of the United States of America *100*, 12009-12014.

Linke, K., Mace, P.D., Smith, C.A., Vaux, D.L., Silke, J., and Day, C.L. (2008). Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. Cell death and differentiation *15*, 841-848.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43-52.

Liu, S., Dontu, G., and Wicha, M.S. (2005). Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast cancer research : BCR *7*, 86-95.

Loeffler, M., and Roeder, I. (2002). Tissue stem cells: definition, plasticity, heterogeneity, self-organization and models--a conceptual approach. Cells, tissues, organs *171*, 8-26.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., *et al.* (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics *38*, 431-440.

Lozano, G., and Montes de Oca Luna, R. (1998). MDM2 function. Biochimica et biophysica acta *1377*, M55-59.

Marcel, V., Dichtel-Danjoy, M.L., Sagne, C., Hafsi, H., Ma, D., Ortiz-Cuaran, S., Olivier, M., Hall, J., Mollereau, B., Hainaut, P., *et al.* (2011). Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. Cell death and differentiation *18*, 1815-1824.

Marcel, V., Khoury, M.P., Fernandes, K., Diot, A., Lane, D.P., and Bourdon, J.C. (2013). Detecting p53 isoforms at protein level. Methods in molecular biology *962*, 15-29.

Marchenko, N.D., Zaika, A., and Moll, U.M. (2000). Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. The Journal of biological chemistry *275*, 16202-16212.

Marion, R.M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M.A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature *460*, 1149-1153.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proceedings of the National Academy of Sciences of the United States of America *78*, 7634-7638.

Matlashewski, G., Lamb, P., Pim, D., Peacock, J., Crawford, L., and Benchimol, S. (1984). Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. The EMBO journal *3*, 3257-3262.

Maynard, S., Swistowska, A.M., Lee, J.W., Liu, Y., Liu, S.T., Da Cruz, A.B., Rao, M., de Souza-Pinto, N.C., Zeng, X., and Bohr, V.A. (2008). Human embryonic stem cells have enhanced repair of multiple forms of DNA damage. Stem cells *26*, 2266-2274.

McKay, R., Renfranz, P., and Cunningham, M. (1993). Immortalized stem cells from the central nervous system. Comptes rendus de l'Academie des sciences Serie III, Sciences de la vie *316*, 1452-1457.

McLaren, A. (2001). Ethical and social considerations of stem cell research. Nature *414*, 129-131.

McLure, K.G., and Lee, P.W. (1998). How p53 binds DNA as a tetramer. The EMBO journal *17*, 3342-3350.

Meek, D.W. (1999). Mechanisms of switching on p53: a role for covalent modification? Oncogene *18*, 7666-7675.

Mercer, W.E., Amin, M., Sauve, G.J., Appella, E., Ullrich, S.J., and Romano, J.W. (1990). Wild type human p53 is antiproliferative in SV40-transformed hamster cells. Oncogene *5*, 973-980.

Michalovitz, D., Halevy, O., and Oren, M. (1991). p53 mutations: gains or losses? Journal of cellular biochemistry *45*, 22-29.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. Molecular cell *11*, 577-590.

Mirza, A., Wu, Q., Wang, L., McClanahan, T., Bishop, W.R., Gheyas, F., Ding, W., Hutchins, B., Hockenberry, T., Kirschmeier, P., *et al.* (2003). Global transcriptional program of p53 target genes during the process of apoptosis and cell cycle progression. Oncogene *22*, 3645-3654.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.

Mizuno, H., Spike, B.T., Wahl, G.M., and Levine, A.J. (2010). Inactivation of p53 in breast cancers correlates with stem cell transcriptional signatures. Proceedings of the National Academy of Sciences of the United States of America *107*, 22745-22750.

Moll, U.M., Marchenko, N., and Zhang, X.K. (2006). p53 and Nur77/TR3 - transcription factors that directly target mitochondria for cell death induction. Oncogene *25*, 4725-4743.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Murphy, M., Ahn, J., Walker, K.K., Hoffman, W.H., Evans, R.M., Levine, A.J., and George, D.L. (1999). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes & development *13*, 2490-2501.

Murray-Zmijewski, F., Slee, E.A., and Lu, X. (2008). A complex barcode underlies the heterogeneous response of p53 to stress. Nature reviews Molecular cell biology *9*, 702-712.

Murrell, W., Feron, F., Wetzig, A., Cameron, N., Splatt, K., Bellette, B., Bianco, J., Perry, C., Lee, G., and Mackay-Sim, A. (2005). Multipotent stem cells from adult olfactory mucosa. Developmental dynamics : an official publication of the American Association of Anatomists *233*, 496-515.

Nag, A., Germaniuk-Kurowska, A., Dimri, M., Sassack, M.A., Gurumurthy, C.B., Gao, Q., Dimri, G., Band, H., and Band, V. (2007). An essential role of human Ada3 in p53 acetylation. The Journal of biological chemistry *282*, 8812-8820.

Neganova, I., and Lako, M. (2008). G1 to S phase cell cycle transition in somatic and embryonic stem cells. Journal of anatomy *213*, 30-44.

Niculescu, A.B., 3rd, Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S.I. (1998). Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Molecular and cellular biology *18*, 629-643.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & development *12*, 2048-2060.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nature genetics *24*, 372-376.

Nortamo, P., Salcedo, R., Timonen, T., Patarroyo, M., and Gahmberg, C.G. (1991). A monoclonal antibody to the human leukocyte adhesion molecule intercellular adhesion molecule-2. Cellular distribution and molecular characterization of the antigen. Journal of immunology *146*, 2530-2535.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature *344*, 503-508.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313-317.

Oliner, J.D., Pietenpol, J.A., Thiagalingam, S., Gyuris, J., Kinzler, K.W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-860.

Olivier, M., Hollstein, M., and Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harbor perspectives in biology *2*, a001008.

Oren, M. (1999). Regulation of the p53 tumor suppressor protein. The Journal of biological chemistry *274*, 36031-36034.

Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in

signal-mediated nuclear protein export. Science *278*, 141-144.

Payne, C.J., and Braun, R.E. (2008). Human adult testis-derived pluripotent stem cells: revealing plasticity from the germline. Cell stem cell *3*, 471-472.

Phinney, D.G., and Prockop, D.J. (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem cells *25*, 2896-2902.

Picksley, S.M., and Lane, D.P. (1993). The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? BioEssays : news and reviews in molecular, cellular and developmental biology *15*, 689-690.

Piette, J., Neel, H., and Marechal, V. (1997). Mdm2: keeping p53 under control. Oncogene *15*, 1001-1010.

Polley, S., Guha, S., Roy, N.S., Kar, S., Sakaguchi, K., Chuman, Y., Swaminathan, V., Kundu, T., and Roy, S. (2008). Differential recognition of phosphorylated transactivation domains of p53 by different p300 domains. Journal of molecular biology *376*, 8-12.

Pommier, Y., Leo, E., Zhang, H., and Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chemistry & biology *17*, 421-433.

Priest, C., Prives, C., and Poyurovsky, M.V. (2010). Deconstructing nucleotide binding activity of the Mdm2 RING domain. Nucleic acids research *38*, 7587-7598.

Qin, H., Yu, T., Qing, T., Liu, Y., Zhao, Y., Cai, J., Li, J., Song, Z., Qu, X., Zhou, P., *et al.* (2007). Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. The Journal of biological chemistry *282*, 5842-5852.

Quante, T., Otto, B., Brazdova, M., Kejnovska, I., Deppert, W., and Tolstonog, G.V. (2012). Mutant p53 is a transcriptional co-factor that binds to G-rich regulatory regions of active genes and generates transcriptional plasticity. Cell cycle *11*, 3290-3303.

Rajendra, R., Malegaonkar, D., Pungaliya, P., Marshall, H., Rasheed, Z., Brownell, J., Liu, L.F., Lutzker, S., Saleem, A., and Rubin, E.H. (2004). Topors functions as an E3 ubiquitin ligase with specific E2 enzymes and ubiquitinates p53. The Journal of biological chemistry *279*, 36440-36444.

Rallapalli, R., Strachan, G., Cho, B., Mercer, W.E., and Hall, D.J. (1999). A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. The Journal of biological chemistry *274*, 8299-8308.

Reddi, A.H. (1995). Bone morphogenetic proteins, bone marrow stromal cells, and mesenchymal stem cells. Maureen Owen revisited. Clinical orthopaedics and related research, 115-119.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nature reviews Molecular cell biology *9*, 402-412.

Robinson, M., Jiang, P., Cui, J., Li, J., Wang, Y., Swaroop, M., Madore, S., Lawrence, T.S., and Sun, Y. (2003). Global genechip profiling to identify genes responsive to p53-induced

growth arrest and apoptosis in human lung carcinoma cells. Cancer biology & therapy *2*, 406-415.

Rodriguez, M.S., Desterro, J.M., Lain, S., Midgley, C.A., Lane, D.P., and Hay, R.T. (1999). SUMO-1 modification activates the transcriptional response of p53. The EMBO journal *18*, 6455-6461.

Roemer, K. (1999). Mutant p53: gain-of-function oncoproteins and wild-type p53 inactivators. Biological chemistry *380*, 879-887.

Roth, J., Dobbelstein, M., Freedman, D.A., Shenk, T., and Levine, A.J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. The EMBO journal *17*, 554-564.

Sabapathy, K., Klemm, M., Jaenisch, R., and Wagner, E.F. (1997). Regulation of ES cell differentiation by functional and conformational modulation of p53. The EMBO journal *16*, 6217-6229.

Saito, S., Yamaguchi, H., Higashimoto, Y., Chao, C., Xu, Y., Fornace, A.J., Jr., Appella, E., and Anderson, C.W. (2003). Phosphorylation site interdependence of human p53 post-translational modifications in response to stress. The Journal of biological chemistry *278*, 37536-37544.

Sakaguchi, K., Saito, S., Higashimoto, Y., Roy, S., Anderson, C.W., and Appella, E. (2000). Damage-mediated phosphorylation of human p53 threonine 18 through a cascade mediated by a casein 1-like kinase. Effect on Mdm2 binding. The Journal of biological chemistry *275*, 9278-9283.

Sakaguchi, K., Sakamoto, H., Lewis, M.S., Anderson, C.W., Erickson, J.W., Appella, E., and Xie, D. (1997). Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53. Biochemistry *36*, 10117-10124.

Sakamuro, D., Sabbatini, P., White, E., and Prendergast, G.C. (1997). The polyproline region of p53 is required to activate apoptosis but not growth arrest. Oncogene *15*, 887-898.

Santibanez-Koref, M.F., Birch, J.M., Hartley, A.L., Jones, P.H., Craft, A.W., Eden, T., Crowther, D., Kelsey, A.M., and Harris, M. (1991). p53 germline mutations in Li-Fraumeni syndrome. Lancet *338*, 1490-1491.

Sax, J.K., Stoddard, A., Murphy, M.E., Chodosh, L., and El-Deiry, W.S. (2003). Microarray expression profiling of p53-dependent transcriptional changes in an immortalized mouse embryo fibroblast cell line. Cancer biology & therapy *2*, 416-430.

Schon, O., Friedler, A., Bycroft, M., Freund, S.M., and Fersht, A.R. (2002). Molecular mechanism of the interaction between MDM2 and p53. Journal of molecular biology *323*, 491-501.

Scian, M.J., Stagliano, K.E., Deb, D., Ellis, M.A., Carchman, E.H., Das, A., Valerie, K., Deb, S.P., and Deb, S. (2004). Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes. Oncogene *23*, 4430-4443.

Sedlacek, Z., Kodet, R., Kriz, V., Seemanova, E., Vodvarka, P., Wilgenbus, P., Mares, J., Poustka, A., and Goetz, P. (1998). Two Li-Fraumeni syndrome families with novel germline p53 mutations: loss of the wild-type p53 allele in only 50% of tumours. British journal of cancer *77*, 1034-1039.

Senturk, S., Yao, Z., Camiolo, M., Stiles, B., Rathod, T., Walsh, A.M., Nemajerova, A., Lazzara, M.J., Altorki, N.K., Krainer, A., *et al.* (2014). p53Psi is a transcriptionally inactive p53 isoform able to reprogram cells toward a metastatic-like state. Proceedings of the National Academy of Sciences of the United States of America *111*, E3287-3296.

Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L., Lindeman, G.J., and Visvader, J.E. (2006). Generation of a functional mammary gland from a single stem cell. Nature *439*, 84-88.

Shaulsky, G., Goldfinger, N., and Rotter, V. (1991). Alterations in tumor development in vivo mediated by expression of wild type or mutant p53 proteins. Cancer research *51*, 5232-5237.

Shi, D., Pop, M.S., Kulikov, R., Love, I.M., Kung, A.L., and Grossman, S.R. (2009). CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proceedings of the National Academy of Sciences of the United States of America *106*, 16275-16280.

Shieh, S.Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes & development *14*, 289-300.

Shvarts, A., Bazuine, M., Dekker, P., Ramos, Y.F., Steegenga, W.T., Merckx, G., van Ham, R.C., van der Houven van Oordt, W., van der Eb, A.J., and Jochemsen, A.G. (1997). Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. Genomics *43*, 34-42.

Shvarts, A., Steegenga, W.T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R.C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A.J., *et al.* (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. The EMBO journal *15*, 5349-5357.

Sieber-Blum, M., and Hu, Y. (2008). Epidermal neural crest stem cells (EPI-NCSC) and pluripotency. Stem cell reviews *4*, 256-260.

Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer research *60*, 6788-6793.

Singh, R.K., Iyappan, S., and Scheffner, M. (2007). Hetero-oligomerization with MdmX rescues the ubiquitin/Nedd8 ligase activity of RING finger mutants of Mdm2. The Journal of biological chemistry *282*, 10901-10907.

Solozobova, V., and Blattner, C. (2010). Regulation of p53 in embryonic stem cells. Experimental cell research *316*, 2434-2446.

Solozobova, V., Rolletschek, A., and Blattner, C. (2009). Nuclear accumulation and activation of

p53 in embryonic stem cells after DNA damage. BMC cell biology *10*, 46.

Soussi, T., and Beroud, C. (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. Nature reviews Cancer *1*, 233-240.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58-62.

Steinmeyer, K., and Deppert, W. (1988). DNA binding properties of murine p53. Oncogene *3*, 501-507.

Stommel, J.M., Marchenko, N.D., Jimenez, G.S., Moll, U.M., Hope, T.J., and Wahl, G.M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. The EMBO journal *18*, 1660-1672.

Stoscheck, C.M. (1990). Quantitation of protein. Methods in enzymology *182*, 50-68.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861-872.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Tang, H.Y., Zhao, K., Pizzolato, J.F., Fonarev, M., Langer, J.C., and Manfredi, J.J. (1998). Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. The Journal of biological chemistry *273*, 29156-29163.

Tanimura, S., Ohtsuka, S., Mitsui, K., Shirouzu, K., Yoshimura, A., and Ohtsubo, M. (1999). MDM2 interacts with MDMX through their RING finger domains. FEBS letters *447*, 5-9.

Thomson, J.A., and Odorico, J.S. (2000). Human embryonic stem cell and embryonic germ cell lines. Trends in biotechnology *18*, 53-57.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology *28*, 511-515.

Ulloa-Montoya, F., Kidder, B.L., Pauwelyn, K.A., Chase, L.G., Luttun, A., Crabbe, A., Geraerts, M., Sharov, A.A., Piao, Y., Ko, M.S., *et al.* (2007). Comparative transcriptome analysis of embryonic and adult stem cells with extended and limited differentiation capacity. Genome biology *8*, R163.

van der Flier, L.G., and Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annual review of physiology *71*, 241-260.

Vassilev, L.T. (2004). Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. Cell cycle *3*, 419-421.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.

Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., and Debussche, L. (1998). The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. The EMBO journal *17*, 4668-4679.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nature reviews Cancer *2*, 594-604.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Walerych, D., Kudla, G., Gutkowska, M., Wawrzynow, B., Muller, L., King, F.W., Helwak, A., Boros, J., Zylicz, A., and Zylicz, M. (2004). Hsp90 chaperones wild-type p53 tumor suppressor protein. The Journal of biological chemistry *279*, 48836-48845.

Walerych, D., Napoli, M., Collavin, L., and Del Sal, G. (2012). The rebel angel: mutant p53 as the driving oncogene in breast cancer. Carcinogenesis *33*, 2007-2017.

Walker, K.K., and Levine, A.J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proceedings of the National Academy of Sciences of the United States of America *93*, 15335-15340.

Wang, X.W., Zhan, Q., Coursen, J.D., Khan, M.A., Kontny, H.U., Yu, L., Hollander, M.C., O'Connor, P.M., Fornace, A.J., Jr., and Harris, C.C. (1999). GADD45 induction of a G2/M cell cycle checkpoint. Proceedings of the National Academy of Sciences of the United States of America *96*, 3706-3711.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature *448*, 318-324.

Wobus, A.M. (2001). Potential of embryonic stem cells. Molecular aspects of medicine *22*, 149-164.

Wolf, D., Harris, N., Goldfinger, N., and Rotter, V. (1985). Isolation of a full-length mouse cDNA clone coding for an immunologically distinct p53 molecule. Molecular and cellular biology *5*, 127-132.

Wolf, D., Harris, N., and Rotter, V. (1984). Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell *38*, 119-126.

Wolff, B., Sanglier, J.J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chemistry & biology *4*, 139-147.

Wu, D., Zhang, Z., Chu, H., Xu, M., Xue, Y., Zhu, H., and Zhang, Z. (2013). Intron 3 sixteen base pairs duplication polymorphism of p53 contributes to breast cancer susceptibility: evidence from meta-analysis. PloS one *8*, e61662.

Xing, J., Sheppard, H.M., Corneillie, S.I., and Liu, X. (2001). p53 Stimulates TFIID-TFIIA-promoter complex assembly, and p53-T antigen complex inhibits TATA binding protein-TATA interaction. Molecular and cellular biology *21*, 3652-3661.

Xiong, S., Van Pelt, C.S., Elizondo-Fraire, A.C., Liu, G., and Lozano, G. (2006). Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. Proceedings of the National Academy of Sciences of the United States of America *103*, 3226-3231.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701-704.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell *118*, 83-97.

Yalow, R.S., and Berson, S.A. (1960). Immunoassay of endogenous plasma insulin in man. The Journal of clinical investigation *39*, 1157-1175.

Yamanaka, S. (2009). A fresh look at iPS cells. Cell *137*, 13-17.

Yamasaki, S., Yagishita, N., Nishioka, K., and Nakajima, T. (2007). The roles of synoviolin in crosstalk between endoplasmic reticulum stress-induced apoptosis and p53 pathway. Cell cycle *6*, 1319-1323.

Yan, C., Lu, D., Hai, T., and Boyd, D.D. (2005). Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination. The EMBO journal *24*, 2425-2435.

Yan, H., Solozobova, V., Zhang, P., Armant, O., Kuehl, B., Brenner-Weiss, G., and Blattner, C. (2015). p53 is active in murine stem cells and alters the transcriptome in a manner that is reminiscent of mutant p53. Cell death & disease *6*, e1662.

Yang, W., Rozan, L.M., McDonald, E.R., 3rd, Navaraj, A., Liu, J.J., Matthew, E.M., Wang, W., Dicker, D.T., and El-Deiry, W.S. (2007). CARPs are ubiquitin ligases that promote MDM2-independent p53 and phospho-p53ser20 degradation. The Journal of biological chemistry *282*, 3273-3281.

Yin, Y., Stephen, C.W., Luciani, M.G., and Fahraeus, R. (2002). p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nature cell biology *4*, 462-467.

Ying, Q.L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.

Zauli, G., Rimondi, E., Corallini, F., Fadda, R., Capitani, S., and Secchiero, P. (2007). MDM2 antagonist Nutlin-3 suppresses the proliferation and differentiation of human pre-osteoclasts through a p53-dependent pathway. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research *22*, 1621-1630.

Zhang, J., Sprung, R., Pei, J., Tan, X., Kim, S., Zhu, H., Liu, C.F., Grishin, N.V., and Zhao, Y. (2009). Lysine acetylation is a highly abundant and evolutionarily conserved modification in Escherichia coli. Molecular & cellular proteomics : MCP *8*, 215-225.

Zhang, P., Elabd, S., Hammer, S., Solozobova, V., Yan, H., Bartel, F., Inoue, S., Henrich, T., Wittbrodt, J., Loosli, F., *et al.* (2015). TRIM25 has a dual function in the p53/Mdm2 circuit. Oncogene.

Zhang, X., Krutchinsky, A., Fukuda, A., Chen, W., Yamamura, S., Chait, B.T., and Roeder, R.G. (2005). MED1/TRAP220 exists predominantly in a TRAP/ Mediator subpopulation enriched in RNA polymerase II and is required for ER-mediated transcription. Molecular cell *19*, 89-100.

Zhang, Y., Wang, J.S., Chen, L.L., Zhang, Y., Cheng, X.K., Heng, F.Y., Wu, N.H., and Shen, Y.F. (2004). Repression of hsp90beta gene by p53 in UV irradiation-induced apoptosis of Jurkat cells. The Journal of biological chemistry *279*, 42545-42551.

Zhu, J., Jiang, J., Zhou, W., Zhu, K., and Chen, X. (1999). Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene *18*, 2149-2155.

Zhu, K., Zhao, J., Lubman, D.M., Miller, F.R., and Barder, T.J. (2005). Protein pI shifts due to posttranslational modifications in the separation and characterization of proteins. Analytical chemistry *77*, 2745-2755.

# 6. ABBREVIATIONS

| Abbreviation | Full name |
|---|---|
| A | Ampere |
| Ac | Acetylation |
| AFP | Alphafeto protein |
| AP | Alkaline phosphate |
| APS | Ammonium persulfate |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CHAPS | 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate |
| $C_T$ | Cycle threshold |
| C-terminus | Carboxyl-terminus |
| ℃ | Degrees Celcius |
| d | Day |
| DBD | DNA binding domain |
| DMEM | Dulbecco’s modified eagle medium |
| DMSO | Dimthysulfoxide |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTPs | Deoxyribonucleotides |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediamine tetraacetic acid |
| EMT | Epithelial-mesenchymal-transition |
| ESCs | Embryonic stem cells |
| et al. | Et alii, and others |
| Eto | Etoposide |
| f.c. | Final concentration |

| | |
|---|---|
| FDA | Food and Drug Administration |
| GOF | gain-of-function |
| h | Hour |
| HAT | histone acetyltransferases |
| HDAC1 | histone deacetylase 1 |
| HEPES | 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid |
| HRP | Horseradish peroxidase |
| i.e. | id ist, that is |
| IEF | Isoelectric focusing |
| iPSCs | Induced pluripotent stem cells |
| kDa | kilo Dalton |
| KO | knockout |
| L | Liter |
| LIF | Leukemia inhibitory factor |
| LMB | Leptomysin B |
| M | Molar |
| m | Milli |
| MDM2 | mouse double minute 2 homolog |
| MDMX | mouse double minute 4 homolog |
| Me | Methylation |
| MEFs | Mouse embryonic fibroblast |
| mESCs | Mouse embryonic stem cells |
| MET | Mesenchymal-epithelial-transition |
| min | Minute |
| µl | Microliter |
| ml | Milliliter |
| MOMP | Mitochondrial outer membrane permeabilization |
| mRNA | Messenger RNA |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |

| | |
|---|---|
| n | Nano |
| NA | Nicotinamide |
| NEAA | Non-essential amino acid |
| NEM | N-Ethylmaleimide |
| NES | Nuclear export signal |
| NL | Non-linear |
| NLS | Nuclear localization signal |
| NP-40 | Nonodet-P40 |
| N-terminus | Amino-terminus |
| o.n. | overnight |
| P | phosphorylation |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS(T) | Phosphate buffered saline (Tween20) |
| PCR | Polymerase chain reaction |
| pH | Potential of hydrogen |
| pI | Isoelectric point |
| PMSF | Phenylmthanesulfonyl fluoride |
| PRD | Proline rich domain |
| PTMs | Posttranslational modifications |
| PVDF | Polyvinylidene difluoride |
| qRT-PCR | Quantitative Real-time PCR |
| RE | Responsive element |
| REG | Regulatory domain |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcription PCR |
| sec | Second |
| SDS | Sodium Dodecyl Sulfonate |

| | |
|---|---|
| siRNA | Small interfering RNA |
| SUMO | Small ubiquitin-like modifier |
| TAD | Transactivation domain |
| TAE | Tris Acetate-EDTA buffer |
| TAF9 | TBP associated factor 9 |
| TBP | TATA binding protein |
| TBS(T) | Tris buffered saline (Tween20) |
| TCA | Trichloroacetic acid |
| TSA | Trichostatin A |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| TET | Tetramerization domain |
| TM | Tris magnesium sulfate |
| Tris | Tris(hydroxymethyl)aminometane |
| UV | Ultraviolet |
| V | Volt |
| W | Walt |
| w/o | without |

# 7. PUBLICATION LIST DURING PhD

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Zhang, P., Elabd, S., Hammer, S., Solozobova, V., **Yan, H.**, Bartel, F., Inoue, S., Henrich, T., Wittbrodt, J., Loosli, F., *et al.* (2015). TRIM25 has a dual function in the p53/Mdm2 circuit. Oncogene. [Epub ahead of print]

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