P53 regulation and activity in mouse embryonic stem cells

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

der Fakultät für Chemie und Biowissenschaften des
Karlsruher Institut für Technologie (KIT) - Universitätsbereich
genehmigte

DISSERTATION

von

Valeriya Solozobova

Aus Surgut - Russland

Dekan/Dean: Prof. Dr. Stefan Bräse

Referent/Referent: PD Dr. Christine Blattner

Koreferent/Co-referent: Prof. Dr. Doris Wedlich

Tag der mündlichen Prüfung/
Day of the oral exam: 14/07/2010

Ich erkläre, dass ich diese Dissertation selbständing angefertig habe. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet.

I hereby declare that this dissertation is my own independent work. I have only used the given sources and materials and I have cited others' work appropriately.

Valeriya Solozobova
Karlsruhe 10.05.2010

Zusammenfassung

P53 ist ein Tumorsuppressor-Protein. Als Reaktion auf verschiedene Arten von Zellstress aktiviert P53 zelluläre Programme, die den Erhalt der genetischen Stabilität ermöglichen, wie zum Beispiel Apoptose, Zellzyklus-Arrest und DNA-Reparatur. Kürzlich wurde entdeckt, dass P53 als Antwort auf DNA-Schäden auch die Differenzierung von embryonischen Stammzellen auslösen kann und dadurch Zellen mit geschädigter Erbinformation vom Pool der Zellen mit Potenzial zur unbegrenzten Selbsterneuerung entfernt. Seither besteht ein hohes Interesse an der Rolle von P53 in embryonischen und weiteren Stammzellen. Es ist jedoch nur wenig über seine Regulierung in diesen Zelltypen bekannt. Das Ziel dieser Arbeit war, die Regulierung von P53 in embryonischen Stammzellen und seine Aktivierung als Antwort auf DNA-Schäden zu untersuchen.

Die erhaltenen Daten zeigen, dass P53 hauptsächlich im Zytoplasma von ungeschädigten ES-Zellen lokalisiert ist. Dessen ungeachtet wurde festgestellt, dass P53 in ES-Zellen als Reaktion auf DNA-Schäden auf der Transkriptionsebene aktiv wird, was einer Zunahme der Menge an P53 im Zellkern von DNA-geschädigten ES-Zellen entspricht.

Embryonische Stammzellen enthalten eine relativ hohe Menge an P53-Protein und P53-mRNA. Nach der Differenzierung wird P53 rasch herunter reguliert. Die hohe Menge an P53 in undifferenzierten ES-Zellen kann nicht durch eine erhöhte Stabilität des P53-Proteins erreicht werden. Tatsächlich wird P53 in ES-Zellen sogar schneller abgebaut als zum Beispiel in embryonischen Mäusefibroblasten. Die Translationsrate von P53 ist dagegen in ES-Zellen höher als in ausdifferenzierten Zellen. Diese verstärkte Translation könnte durch die erhöhte Aktivität des L26-Proteins sowie eine geringere Menge an den MicroRNAs miRNA125a und miRNA125b in ES-Zellen verursacht werden, da eine Überexpression von L26 die Menge an P53 erhöhte wohingegen seine Herunterregulierung oder eine Überexpression von miRNA125a oder miRNA125b die P53-Menge verringerte.

Trotz seiner Lokalisierung im Zytoplasma wird P53 in ES-Zellen von der E3-Ligase MDM2, die hauptsächlich im Zellkern lokalisiert ist, zum Abbau im 26S-Proteasom markiert. Dieser Vorgang wird zusätzlich durch das Deubiquitinierungs-

Enzym Hausp kontrolliert. Eine andere E3-Ligase, PirH2, scheint dagegen weniger wichtig für die Kontrolle von P53 in ES-Zellen zu sein. Dies deutet darauf hin dass, im Gegensatz zu den Unterschieden in der Kontrolle der Translation, der Abbau von P53 in embryonischen Stammzellen ähnlich erfolgt wie in ausdifferenzierten Zellen.

Abstract

P53 is a tumour suppressor protein. After various types of cellular stress p53 activates programs that allow maintenance of genomic stability, such as apoptosis, cell cycle arrest and DNA repair. Recently it was found that p53 might also activate differentiation of embryonic stem cells after DNA damage thereby eliminating cells with damaged genomic information from the pool of cells with unlimited self-renewal potential. Since that time the interest in the role of p53 in embryonic and other stem cells is increased. However, not much is known about its regulation in this cell type. The aim of this work was to study the regulation of p53 in embryonic stem cells and its activation in response to DNA damage.

The data show that p53 is predominantly localised in the cytoplasm of undamaged ES cells. Nevertheless, p53 was found that p53 becomes transcriptionally active in ES cells after DNA damage, which corresponds to an increase in the amount of p53 in the nucleus of DNA damaged-ES cells.

Embryonic stem cells contain a relatively high amount of p53 protein and p53 RNA. After differentiation p53 level is rapidly downregulated. The high abundance of p53 in undifferentiated ES cells can not be achieved by enhanced stability of the p53 protein. In fact, it is more rapidly turned-over in ES cells than for example in mouse embryonic fibroblasts. However, p53 translation occurs at a higher rate in ES cells than in differentiated cells. This increased translation might be caused by higher activity of the L26 protein and lower levels of the microRNAs miRNA125a and miRNA125b in ES cells since overexpression of L26 increased p53 abundance whereas its downregulation or overexpression of miRNA125a or 125b decreased p53 abundance.

Despite its cytoplasmic localisation, p53 is still targeted by the E3 ligase MDM2 oncoprotein mostly localized into the nucleus for degradation in 26S proteasomes in embryonic stem cells. This process is further controlled by the deubiquitinating enzyme Hausp. Another E3 ligase, PirH2 appears to be less important for the control of p53 in embryonic stem cells, suggesting that in contrast to many differences in translational control, the degradation pathway for p53 is ES cells is similar to that in differentiated cells.

TABLE OF CONTENTS

Zusammenfassung ……………………………………………………………3
Abstract…………………………………………………………………….. 5
Table of contents…………………………………………………………… 6
Abbreviations……………………………………………………………….9
1 Introduction……………………………….……………………………...12
1.1 Embryonic stem cells…………………………………………..12
1.1.1 Embryonic stem cells and DNA damage………………...13
1.2 The tumour suppressor protein p53…………………………..14
1.2.1 P53 and the DNA damage response……………………...14
1.2.2 Regulation of p53…………………………………………16
1.2.3 P53 in ES cells……………………………………………..20
1.2.3.1 p53 activity and regulation in ES cells after
DNA damage…………………………………..………20
1.2.3.2 p53 and its role in the differentiation of stem cells…21
1.2.3.3 p53 and its role in induced pluripotent cells………..24
1.3 Aim………………………………………………………………27
2 Materials and methods………………..……………………………..28
2.1 Materials………………………………………………………...28
2.1.1 Chemicals and consumables……………………………..28
2.1.2 Bacteria and eukaryotic cell lines…...…………………..…31
2.1.2.1 Bacteria……………………………………….……31
2.1.2.2 Eukaryotic cell lines……………...……….………31
2.1.3 Oligonucleotides…………………...……..………………31
2.1.3.1 Primers for cloning ………………….……………31
2.1.3.2 Sequneces of siRNAs…………………..…………..32
2.1.3.3 Primers for RT-PCR…………………..…………..32
2.1.3.4 Primers for the determination of microRNA-125a
and miRNA-125b expression…………………..…………..33
2.1.4 Primary antibodies………….…………….……………..33
2.1.5 Secondary antibodies……………………………….....35
2.1.6 Enzymes……………………………………………………36

2.1.7 Plasmids……………………………………………………….36

2.2 Methods…………………………….………………………………37

2.2.1 Nucleic acid techniques……..………………………………37

2.2.1.1 Transformation of bacteria…………………….…….37

2.2.1.2 Small-scale purification of plasmid DNA...………….37

2.2.1.3 Large-scale purification of plasmid DNA……..…….38

2.2.1.4 Determination of the plasmid DNA concentration of.39

2.2.1.5 Separation of nucleic acids by agarose gel electrophoresis………………………………………….…….39

2.2.1.6 Extraction of DNA from agarose gel……..………..40

2.2.1.7 Extraction of RNA from eukaryotic cells…..………..40

2.2.1.8 cDNA synthesis……………………………….……….41

2.2.1.9 qRT-PCR…………………………………..………..41

2.2.1.10 cDNA synthesis of microRNAs…………..……….42

2.2.1.11 PCR for determination of microRNA expression.…43

2.2.1.12 Cloning of microRNA-125a and 125b……..……..…43

2.2.1.12.1 PCR……………………..……………………....43

2.2.1.12.2 Restriction digestion………..…………….…..44

2.2.1.12.3 Ligation of DNA fragments…..…………...…...44

2.2.2 Cell culture and transfection methods………..…....…45

2.2.2.1 Culturing of eukaryotic cells………………………..…45

2.2.2.2 Freezing and thawing of the cells……..…………...….46

2.2.2.3 Preparation of plates to culture ES cells…..…..……..46

2.2.2.4 Transfection of ES cells………...…….……………..46

2.2.2.5 Differentiation of ES cells………..…….…………....47

2.2.2.5.1 Differentiation of ES cells by all-*trans*-retinoic acid…………………………………………….47

2.2.2.5.2 Differentiation of ES cells by formation of embryoid bodies…………………….……………….47

2.2.2.6 Irradiation of cells and special treatments..…….……48

2.2.2.7 Alkaline Phosphatase staining………......………48

2.2.3 Protein Methods……,…..,,…………………..……….48

2.2.3.1 Determination of protein localization by

immunofluorescent microscopy……………………………….……..48
2.2.3.2 Preparation of protein lysates from cells……………49
2.2.3.3 Determination of the protein concentration
of cellular lysates………...…………………………………49
2.2.3.4 Separation of proteins by SDS-PAGE……….……..50
2.2.3.5 Western blotting and protein detection……....…….51
2.2.3.6 Immunoprecipitation……………………….....…...51
2.2.3.7 Co-immunoprecipitation………..……………….……52
2.2.3.8 Metabolic labelling…………………………….....…52
2.2.3.9 Ubiquitination Assay…………………………..….....…53
3 Results……………………………………………………………...54
3.1 P53 level and localization in ES cells and during differentiation…..54
3.2 Degradation of the p53 protein in ES cells………………………… 56
3.3 Regulation of p53 in ES cells occurs at the level of RNA stability and translation………………………………………….…………………...63
3.4 Regulation of cytoplasmic localization of p53 in ES cells…………...70
3.5 P53 activity after DNA damage in ES cells……………...…………...73
4 Discussion………………………………………………………………..77
4.1 Regulation of p53 abundance in ES cells……………………………77
4.2 P53 resides in the cytoplasm of ES cells……………….……………...80
4.3 P53 is active in ES cells after DNA damage……………….…………81
4.4 Conclusion………………………………………….…………...83
5 Outlook………………………………………………………………..84
6 References……………………………………………………………85
Curriculum vitae………………………………………………………..99
Publication list………………………………...……………………100
Acknowledgements…………………………..……………………..101

ABBREVIATIONS

ARF-BP1	ARF-binding protein 1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Bcl-2	B-cell leukemia/lymphoma 2
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumine
ºC	Degrees Celsius
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CHX	Cycloheximide
Co-IP	Co-immunoprecipitation
C-terminal	Carboxy- terminal
d	days
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	deoxynucleosides triphosphate
DTT	Dithiothreitol
EB	Embryoid body
ECL	Enhancer of chemioluminescence
EDTA	Ethylenediamine Tetraacetic Acid
ES	Embryonic stem
ESC	embryonic stem cells
et al.	*Et alii*, and others
FBS	Fetal bovine serum
Fig.	Figure
FZK	Forschungszentrum Karlsruhe
g	gram
g	gravity (unit of relative centrifugal force)
h	hour
HAUSP	herpesvirus-associated specific hydrolase
HCl	Hydrochloric acid
HSC	hematopoietic stem cell
HRP	Horseradish peroxidase
ICM	inner cell mass
IF	immunofluorescence
IP	Immunoprecipitation
iPSC	induced puluripotent stem cell

IMDM	Iscove's modified Dulbecco's medium
ITG	Institute of Toxicology and Genetics
IR	ionising radiatiob
KCl	Potassium cloride
kDa	kilodalton
l	liter
LIF	leukemia inhibitory factor
M	molar
µ	micro
m	milli
min	minute
miRNA	microRNA
Mdm2	Murine double minute 2
mRNA	messenger RNA
MSC	mesenchimal stem cells
n	nano
NaCl	Sodium chloride
NLS	Nuclear localisation signal
NP-40	Nonident P-40
N-terminal	Amino- terminal
OD	optical density
ON	overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Posphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGC	primordial germ cells
p	pico
PMSF	phenylmethanesulphonylfluoride
pRb	phoshorylated retinoblastoma
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
Rb	retinoblastoma
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	race per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	real-time polymerase chain reaction
s	second
SD	standard deviation
SDS	Sodium dodecyl sulfate

siRNA	small interfering RNA
SNT	supernatant
SV40	Simian Virus 40
TAE	Tri/acetate/EDTA electrophoresis buffer
Tag	T antigen
TBS	Tris buffer saline
TEMED	tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminometane
U	units
UV	ultraviolet
V	volt
WB	Western blot
w/o	without
v/v	volume on volume
v/w	volume on weight

1. INTRODUCTION

1.1 Embryonic stem cells

Stem cells are present throughout embryonic development as well as in adult organs. There are two broad types of stem cells. The first class are embryonic stem (ES) cells that are isolated from the inner cell mass of blastocyst stage of the embryo, and the second are the adult stem cells that are found in adult tissues. Embryonic stem cells are omnipotent and can differentiate into all tissues of an embryo (Fig.1.1) while tissue stem cells and progenitor cells maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues but also act as a repair system for the body, replenishing specialized cells, after injury.

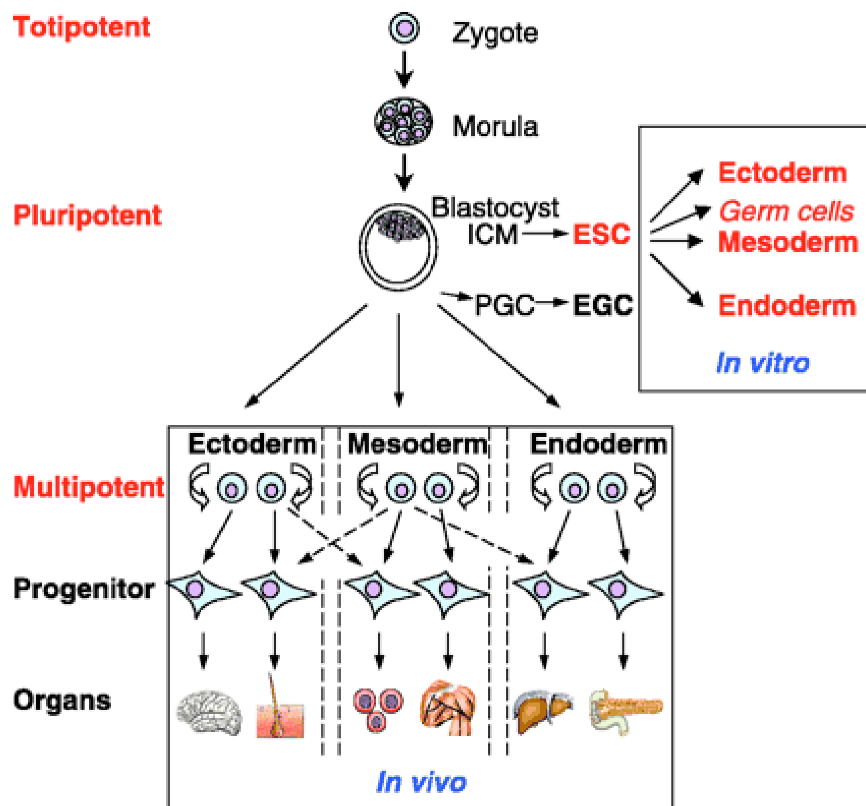

Fig. 1.1: Stem cell hierarchy

1.1: Embryonic stem cells (ESC), derived from the inner cell mass (ICM) of blastocyst, have the developmental capacity to generate a complex organism, to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC) or differentiate *in vitro* into cells of all somatic cell lineages as well as into male and female germ cells (from Biology Online).

EGC – embryonic germ cells.

Coordinated control of stem cell self-renewal and differentiation is a key to maintain homeostasis, and its deregulation contributes to cancer development and other diseases (Reya et al. 2001).

1.1.1 Embryonic stem cells and DNA damage

The maintenance of genomic stability in ES cells must be stringent, because any genetic alterations in those progenitor cells might compromise the genomic stability and functionality of entire cell lineages and whole organism. Therefore, the maintenance of genomic fidelity in ES cells may require additional mechanisms to protect their genomic integrity.

Consistently, the mutation rate and the frequency of mitotic recombination are lower in murine ES cells than in adult somatic cells or mouse embryonic fibroblasts (MEF). For instance, the frequency of spontaneous mutation at the *aprt* gene is around 10 in ESC and 100-fold higher (~10) in MEF (Hong, Cervantes et al. 2007). Similarly, when spontaneous mutation was estimated at the X-chromosome-linked locus *hprt*, it was undetectable in ESC ($<10^{-8}$) and $\sim10^{-5}$ in MEF. Therefore, robust mechanisms counteracting spontaneous mutagenesis may exist in ES cells (Park and Gerson 2005; Lin, et al. 2006; Maynard et al. 2008; Tichy and Stambrook 2008; Frosina 2009).

Using single-cell gel electrophoresis Maynard and coworkers (Maynard et al. 2008) found that human ES cells have more efficient repair mechanisms for different types of DNA damage (generated from ultraviolet (UV)-C, ionizing radiation (IR) or psoralen) than human primary fibroblasts and, with the exception of UV-C damage, HeLa cells. A microarray gene expression analysis showed that the mRNA levels of several DNA repair genes were elevated in human ESC compared with their differentiated forms (such as embryoid bodies). Therefore, multiple DNA repair pathways are over-regulated in human ESC, relative to differentiated human cells (Maynard et al. 2008).

Moreover, the expression of antioxidant and DNA repair genes was reduced and the DNA damage levels increased during spontaneous differentiation of two human ESC lines (Saretzki et al. 2008). Also, the expression of strand break repair genes such

as Rad51 reduces while murine ESC are differentiating (Saretzki et al. 2004; Saretzki et al. 2008; Tichy and Stambrook 2008).

Another mechanism how ES cells maintain genomic integrity is their sensitivity to DNA damage that results in an elevated rate of cell death or differentiation after DNA damage and removes damaged cells from the pool of ES cells (Aladjem et al. 1998; Van Sloun et al. 1999). It is presently unclear why ES cells show this enhanced induction of apoptosis after DNA damage. However, since restoration of a G1/S checkpoint in ES cells protects them from DNA damage-induced apoptosis (Hong and Stambrook 2004) it is speculated that the increased rate of apoptosis in ES cells after DNA damage is due to the lack of an effective cell cycle G1/S checkpoint, which is normally present in somatic cells (Aladjem et al. 1998).

1.2 The tumour suppressor protein p53

The tumour suppressor protein p53 was first described in 1979 (DeLeo et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979) and ten years later it was identified as a tumour suppressor protein (Levine 1990).

Nowadays it is generally accepted that p53 plays a crucial role in the prevention of tumour development. P53 is non-functional or functions incorrectly in most human tumours (reviewed by (Vogelstein et al. 2000)). More than half of human tumours harbour mutations in p53 and in most of the remaining cases the p53 pathway is inactivated by other mechanisms, such as through overproduction of the p53 inhibitor Mdm2 (Toledo and Wahl 2006). The importance of a functional p53 protein is further accentuated by the fact that p53-deficient mice show a very high incidence of multiple, spontaneous tumours which they develop at an early age (Donehower et al. 1992; Donehower et al. 1995). Inherited mutations in the p53 gene lead to the cancer-predisposition disease Li-Fraumeni syndrome (Malkin et al. 1990).

By regulating cellular responses to DNA damage and other forms of genotoxic stress, p53 is a key element in maintaining genomic stability, and this is why it has earned its nickname ‘guardian of the genome’ (Lane 1992).

1.2.1 P53 and the DNA damage response

In response to DNA damage and other forms of cellular stress, the protein levels of p53 are greatly up-regulated and its activity is induced. The p53 protein is normally a very short-lived protein that is rapidly degraded in cellular proteasomes in unperturbed cells (Maltzman and Czyzyk 1984). In response to various types of cellular stress such as DNA damage, hypoxia, oncogene activation or nutrient deprivation, the p53 protein is stabilized and initiates several cellular programs including cell cycle arrest, senescence, apoptosis, DNA repair and differentiation (Levine 1990; Kastan et al. 1991; Lane 1992; Levine 1997; Albrechtsen et al. 1999; Vogelstein et al. 2000; Vousden and Lu 2002; Lin et al. 2005; Moll et al. 2005) An essential feature of p53's activity is p53-controlled transactivation and repression of its target genes, although some effects of p53 are also due to non-transcriptional functions. As an example, induction of p53-dependent apoptosis is not only caused by p53-dependent transcriptional activation of pro-apoptotic genes, as Puma and Noxa, but also by its association with mitochondria, which releases cytochrom C from the intermembrane space as well as by its interaction with Bcl-xL and Bcl-2, which antagonises their anti-apoptotic functions, and with Bak, thereby promoting its pro-apoptotic activity (Vogelstein et al. 2000; Moll et al. 2005; Marchenko et al. 2007; Vousden and Lane 2007).

Cell cycle checkpoints permit repair of damaged DNA before the cell reinitiates replicative DNA synthesis (G1 arrest) or begins mitosis (G2 arrest). The G1 arrest involves p53-dependent transcriptional activation of p21 (el-Deiry et al. 1994). P21 inhibits different complexes of cyclin/cyclin-dependent kinases (cdks) (Cyclin D-Cdk4/6 and Cyclin A, E-Cdk2) that sequentially phosphorylate the retinoblastoma (pRb) protein, thereby resulting in release of the S phase-promoting E2F-1 transcription factor (Brehm et al. 1999) (Fig.1.2).

P53 was shown also to regulate G2/M transition (reviewed in (Taylor and Stark 2001)). P53 initiates G2 cell cycle arrest by blocking Cdc2, cyclin-dependent kinase required for entering mitosis. P53 transcriptionally activates Gadd45, p21 and 14-4-4-s those inhibit Cdc2 (Hermeking et al. 1997; Bunz et al. 1998; Dulic et al. 1998; Zhan et al. 1999; Smits et al. 2000). Additionally, p53 transcriptionally represses cyclin B1 that required for cdc2 activity, and cdc2 itself (Innocente et al. 1999; Taylor et al. 1999; Park et al. 2000).

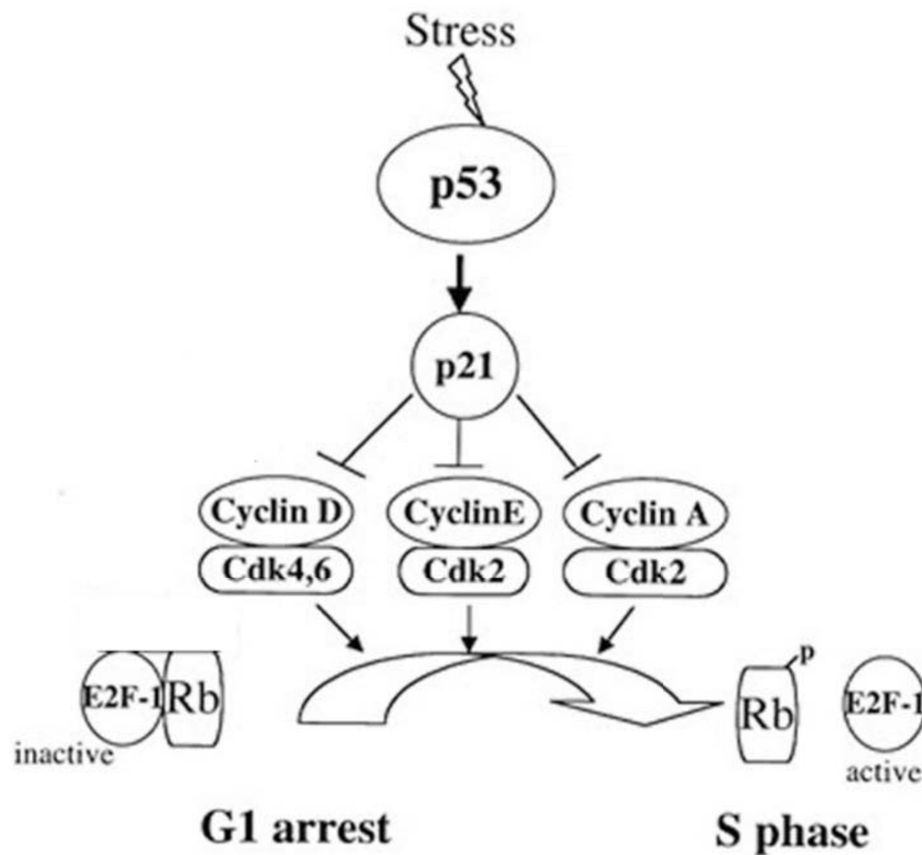

Fig.1.2 G1-cell cycle arrest mediated by p53 (from V.Sionov, 2000, Madame Curie Bioscience Database).

Fig.1.2: The p53-target gene, p21 (waf-1/cip-1), is the key player in G1 arrest. P21 inhibits cyclin-dependent kinases (Cdk) that inactivate retinoblastoma protein (Rb) by phosphorylation. Dephosporylated Rb binds E2F1 causing block of cell cycle progression.

1.2.2 Regulation of p53

As a transcriptional factor that activates genes involved in proliferation arrest and apoptosis, p53 demands a complex network to control and fine-tune its activity (Brooks and Gu 2003; Laptenko and Prives 2006).

The main level at which p53 is regulated is at the level of protein abundance (Ashcroft et al. 1999; Vogelstein et al. 2000). The tight control of cellular p53 levels is primarily achieved through its ubiquitin-mediated proteasomal degradation (Michael and Oren 2003; Brooks and Gu 2006). The ubiquitin-dependent pathway to protein degradation involves the covalent attachment of ubiquitin to substrate proteins to yield ubiquitin-protein conjugates. Ubiquitin is activated initially by ubiquitin-activating enzyme (E1) via formation of a thioester bond with this enzyme. The activated ubiquitin

is then transferred to one of many distinct ubiquitin-conjugating enzymes (E2) by transthiolation. The E2 enzymes catalyze the ubiquitination of substrate proteins either directly or in conjunction with a distinct ubiquitin ligase (E3). The ubiquitination of a substrate protein is followed by degradation of the protein by the proteasome (Hershko and Ciechanover 1998).

The oncogene Mdm2 was found to be the principal regulator of p53 protein. Mdm2 is an E3 ubiquitin ligase and as such it promotes polyubiquitination of p53 and targets the tumour suppressor protein for degradation by the 26S proteasome (Kulikov et al. 2010; Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997; Fang et al. 2000). Importantly, MDM2 itself is the product of a p53-inducible gene (Barak, et al. 1993; Picksley and Lane 1993; Wu and Levine 1997). Thus, the two molecules are linked to each other through an autoregulatory negative feedback loop to maintain low cellular p53 levels in the absence of stress (Fig.1.3).

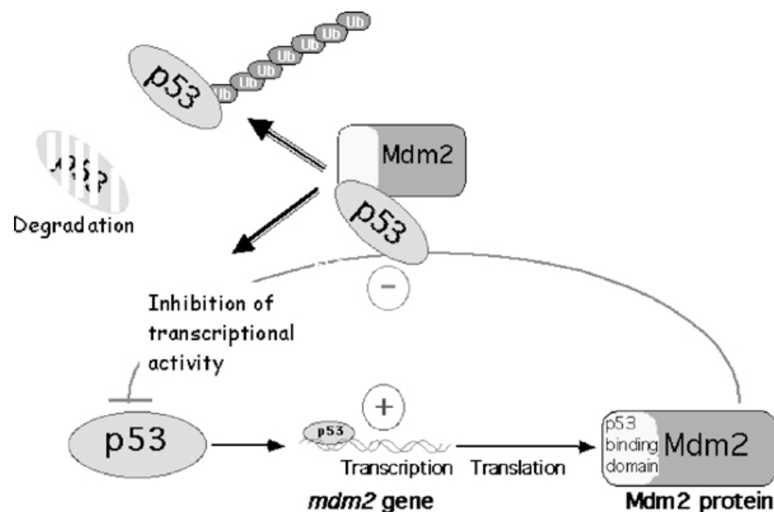

Fig. 1.3: p53 transcriptionally regulates its negative regulator Mdm2 thereby creating autoregulatory feedback loop.

1.3: p53 in response to cell stress activates transcription of its target genes. One of them is *mdm2*, negative regulator of p53. Mdm2 ubiquitinates p53 that results in its degradation. At the same time Mdm2 inhibits transcriptional activity of p53**.**

In addition, Mdm2 reduces p53 transcriptional activity, at least after overexpression (Momand et al. 1992; Finlay 1993; Oliner et al. 1993; Chen et al. 1996). Apart from Mdm2, p53 can also be ubiquitinated by other ubiquitin ligases such as ARF-BP1, Cop1 or PirH2 (reviewed in (Lee and Gu ; Boehme and Blattner 2009)

Interestingly, Mdm2 can both mono- and polyubiquitinate p53, depending on Mdm2 protein levels (Li et al. 2003). Since, ubiquitination is not only associated with protein degradation but with many other cellular processes (Hicke 1997; O'Neill 2009), the amount of ubiquitin molecules that are attached to a given protein and the specific linkage between different ubiquitin molecules as well as with their substrate is important for the consequences resulting from ubiquitination (Kim and Rao 2006). As such, monoubiquitinated forms of p53 are not prone to proteasomal degradation but are rather targeted for export from the nucleus (Lee and Gu, 2010). However, the function of monoubiquitination for the regulation of p53 activity is still inconclusive. Apart from Mdm2, additional enzyme control p53 ubiquitination such as Arf-BP1, Cop1 or PirH2 (Leng et al. 2003; Dornan et al. 2004; Chen et al. 2005), reviewed in (Boehme and Blattner 2009). Ubiquitination of p53 by these enzymes is followed by p53 degradation. Some ubiquitinating enzymes are involved in degradation-independent ubiquitination of p53. One of these enzymes is Ubc13, an E2 ubiquitin-conjugating enzyme that decreases p53 transcriptional activity, attenuates p53-induced apoptosis and increases its localization in the cytoplasm (Laine et al. 2006). Moreover, Ubc13 overexpression increased p53 abundance further supporting that Ubc13-mediated ubiquitination does not target p53 for proteasomal degradation. Additional E3 ubiquitin ligases that are involved in degradation-independent ubiquitination of p53 are WWP1, E4F1 and MSL2 ((Le Cam et al. 2006; Laine and Ronai 2007; Kruse and Gu 2009), reviewed in (Lee and Gu, 2010)). Ubiquitination is a reversible process. As such, ubiquitin molecules can be removed from p53 by deubiquitinating enzymes. The ubiquitin hydrolase HAUSP has been found to deubiquitinate p53 in the nucleus resulting in enhanced p53 stability (Li et al. 2002). HAUSP can further deubiquitinate mono-ubiquitinated p53 in the cytosol and thus affect non-transcriptional functions of p53 (Marchenko and Moll 2007).

Although abundance of p53 is largely controlled at the level of protein stability, several reports have suggested that regulation of p53 translation may also contribute to p53 levels (Chu et al. 1999; Chu and Reyt 1999; Ju et al. 1999; Mazan-Mamczarz et al. 2003; Takagi et al. 2005; Le et al. 2009; Zhang et al. 2009). One of the mechanisms by which p53 translation is controlled is by binding of the ribosomal protein L26 to the 5' UTR of p53 mRNA. This association of L26 with the 5' UTR of p53 mRNA enhances p53 translation, particularly in response to DNA damage (Takagi, Absalon et al. 2005). Recently, it was found that translation of p53 is also controlled by micro-RNAs (Le et

al. 2009; Zhang et al. 2009). Micro-RNAs are a class of small non-coding RNAs (Bartel 2004) that regulate gene expression by inhibiting translation or by reducing RNA stability. The micro-RNAs miRNA-125a and miRNA-125b bind to the 3' UTR of p53 RNA and repress p53 protein synthesis (Le et al. 2009; Zhang et al. 2009). MiRNA-125b-mediated regulation of p53 modulates the rate of apoptosis in human cells and in zebrafish embryos during stress response and development (Le et al. 2009).

P53 activity is furthermore regulated by an array of posttranslational modifications both during normal homeostasis as well as under stress-induced conditions (reviewed in (Zhang and Xiong 2001; Brooks and Gu 2006; Boehme and Blattner 2009; Kruse and Gu 2009; Vousden and Prives 2009). These modifications mostly regulate p53 subcellular localization and DNA-binding and transcriptional activities.

The most widely studied and best-known post-translational modification of p53 is phosphorylation. After DNA damage induced by ionizing radiation or UV light, p53 is phosphorylated at several sites in the N-terminal domain (reviewed in (Appella and Anderson 2001)). A broad range of kinases can modify p53, including ATM/ATR/DNA-PK, and Chk1/Chk2 (Shieh et al. 1997; Shieh et al. 2000; Appella and Anderson 2001).

Histone acetyltransferases provide another important layer of p53 regulation. These enzymes acetylate the C-terminus of p53, frequently after p53 has been phosphorylated at the N-terminus. These modifications enhance binding of p53 to promoters of several p53 target genes and can influence the choice of promoters to which p53 binds. Moreover, by competing with lysines that are can be modified by ubiquitin in the course of p53 degradation, acetyltransferases can modulate p53 stability. Modifications of p53 by SUMO and Nedd8 also regulate p53 function, however the exact consequences of these modifications are still unclear (reviewed in (Kruse and Gu 2009)), particularly as some studies report that sumoylation of p53 promotes its transcriptional activity (Melchior and Hengst 2002) whereas others demonstrate that sumoylation promotes cytoplasmic localization of p53 (Carter et al. 2007). Mdm2-mediated neddylation (Xirodimas et al. 2004) and FBXO11-mediated neddylation (Abida et al. 2007) seem to inhibit p53-mediated transcriptional activation.

The p53 protein possesses nuclear import and export sequences. For many of its functions, p53 needs to be localized in the nucleus and the activity of p53 is regulated

by both nuclear import and nuclear export (Stommel et al. 1999), for a review, see (Vousden and Woude 2000)). Many proteins that interact with p53 may also regulate its intracellular localisation (reviewed by (Vousden and Woude 2000; Vousden and Lu 2002)).

1.2.3 P53 in ES cells

1.2.3.1 P53 activity and regulation in ES cells after DNA damage

P53 is an essential part of the system that protects cells from genomic instability. ES cells are pluripotent, permanent cells, capable of contributing to normal embryogenesis and may therefore have a strong interest in maintaining genomic integrity. Therefore, p53 might be particularly important for ES cells.

Several studies addressed the function and activity of p53 in ES cells in the past, however, with contrary and contradictory information. Aladjem et al. reported that p53 failed to activate a stress response in ES cells after treatment with PALA, IR or adriamycin (Aladjem et al. 1998). They found that p53 is mainly localised in the cytoplasm of ESCs and concluded that p53 may be non-functional because it is sequestered from its target genes in the nucleus. Also some other authors reported that p53 is not able to induce transactivation of its target genes, *p21*, *bax*, and *mdm2* (Hong and Stambrook 2004; Qin et al. 2007; Chuykin et al. 2008). Nevertheless, other authors observed transactivation of the p53 target genes *p21, mdm2, puma and noxa* (Lin et al. 2005; Qin et al. 2007; Filion et al. 2009) in human and mouse ES cells in response to various DNA-damage causing agents. Moreover, the usage of nutlin, a small molecule that prevents the interaction of p53 and Mdm2 and thus releases p53 from Mdm2-mediated control (Vassilev et al. 2004), caused p53 accumulation in ES cells and activation of p21 and Mdm2 (Maimets et al. 2008).

On the other hand fail of ES cells to implement a p53-dependent cell cycle arrest at the G1/S boundary of the cell cycle (Aladjem et al. 1998; Hong and Stambrook 2004; Filion et al. 2009). It is thought that this absence of a G1 arrest is responsible for the extremely high sensitivity of mouse ES cells to UV irradiation. However, this sensitivity of ES cells towards DNA damage may be independent of p53. Corbet et al.

showed that the presence of p53 reduces the colony-forming ability of mouse ES cells in response to g-irradiation however they did not observed significant reduction of apoptosis in p53-null cells (Corbet et al. 1999). This result is consistent with the observation of Aladjem et al., who also claim p53-independent apoptosis after DNA damage in ES cells (Aladjem et al. 1998). Despite these observations of p53-independent apoptosis in ES cells increased the absence of p53 the proliferation rate and reduced apoptosis in ES cells (Sabapathy et al. 1997; Qin et al. 2007). The situation regarding p53 activity in ES cells becomes even more complex as the activity of p53 may differ between human and murine ES cells. For example was the p53 inhibitor a-pifithrin able to inhibit apoptosis in murine but not in human ES cells (Qin et al. 2007).

1.2.3.2 P53 and its role in the differentiation of stem cells

More recently, it became apparent that p53 may control self renewal and differentiation of ES cells and tissue stem cells. In 2005 Lin et al. found that p53 induced differentiation of ES cells in response to DNA damage by suppressing expression of the stem cell marker *nanog* (Lin et al. 2005). Nanog is a homeodomain protein that is required for the maintenance of self-renewal and an undifferentiated state of ES cells and its expression is rapidly down-regulated during differentiation (Chambers et al. 2003; Mitsui et al. 2003). The *nanog* promoter contains two binding sites for p53. Moreover, reducing the amount of p53 in ES cells reduced the amount of spontaneous differentiation of human ES cells (Nichols et al. 1998; Loh et al. 2006). In addition, the rates of spontaneous and induced differentiation were reduced in ES cells with a genetic deletion of both alleles of p53 (Qin et al. 2007).

Similarly, treatment of cells with Nutlin, a small compound that leads to the stabilisation and accumulation of p53 (Vassilev et al. 2004) induced differentiation of human ES cells. However, in contrast to the report by Lin and coworkers, Maimets and co-workers did not observe binding of p53 to the *nanog* promoter (Maimets et al. 2008). Instead, they regarded a p53-induced G1-cell cycle arrest as the starting point of differentiation. This idea is mostly based on the rationale that sodium butyrate, an activator of p21 that is independent of p53, also caused differentiation of human ES cells (Maimets et al. 2008). However, since ES cells are usually refractory to the

induction of p21 at the protein level, it is unclear how the G1 arrest can be implemented at the first place.

In contrast to these reports demonstrating differentiation-inducing activities of p53 is a recent report that shows that p53 possesses anti-differentiation activities in murine ES cells. Lee and co-workers observed beside the induction of growth arrest and apoptosis-inducing target genes (*p21*, *mdm2*, *pirh2*, *puma*, *noxa*) induction of components of the Wnt signalling pathway (Wnt ligands (Wnt3, Wnt3a, Wnt8a, Wnt8b, and Wnt9a), Wnt receptors (Fzd1, Fzd2, Fzd6, Fzd8, Fzd10), and a member of Lef1/Tcf transcription complex (Lef1) in response to UV-irradiation or treatment with adriamycin (Lee et al. 2010). Importantly, the induction of Wnt ligand genes by adriamycin, nutlin and UV were p53 dependent because the reduction of p53 by two short interference RNAs (siRNAs) decreased the fold induction. It had been shown before that Wnt pathway inhibits differentiation of mouse ES cells and is therefore extremely important for maintaining their pluripotency (Dravid et al. 2005; Ogawa et al. 2006). Therefore, study of Lee et al. provided new evidence of anti-differentiation activity of p53 in response to DNA damage (Lee et al. 2010).

The dual role of p53 in differentiation of ES cells might be explained next way: when DNA damage occurs, p53 activity removes the unhealthy cells from the stem-cell pool by promoting programmed cell death or differentiation by repression of *nanog.* At the same time, p53 activates the Wnt pathway to inhibit the differentiation of others, healthy embryonic stem cells to maintain a population for the development of the organism (Fig.1.4).

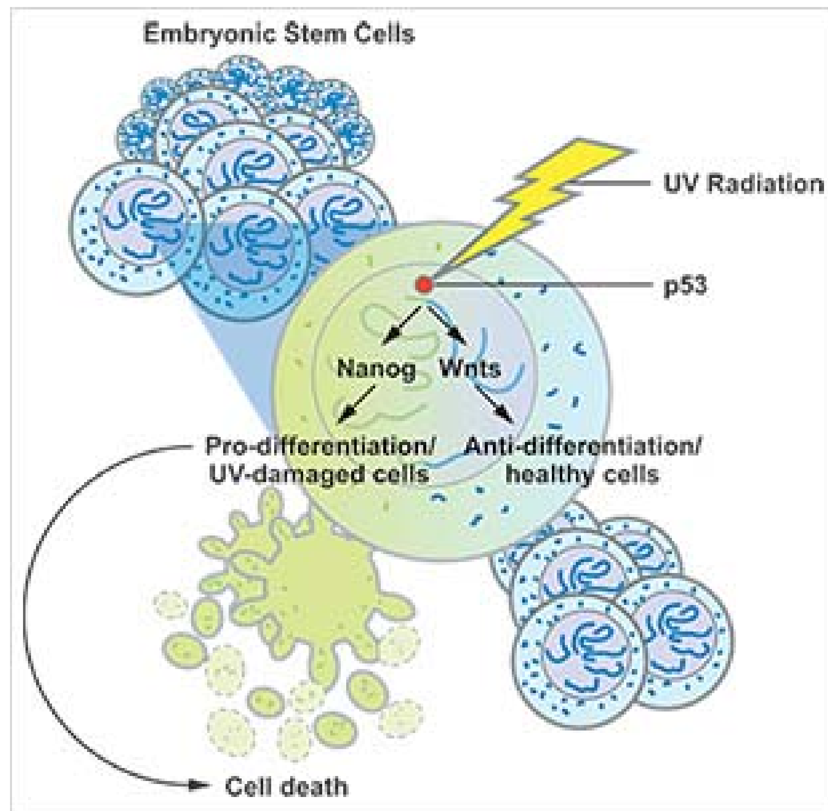

Fig.1.4 Pro- and anti-differentiation activity of p53 (from Summary in Journals of Center of cancer Research, 12/2009).

1.4: After treatments that cause DNA damage, for example UV radiation, p53 induces cell death differentiation of embryonic stem cells with damaged DNA information, whereas in healthy cells it maintains undifferentiated state.

P53 was also found to be an essential regulator of differentiation and self-renewal of tissue stem cells. It has, for example, been shown that the number of proliferating cells in the subventricular zone was higher in the brain of p53-null mice than in the brain of mice with wild-type p53. Moreover, isolated neural stem cells or from the olfactory bulb from p53-null mice produced a higher number of neurospheres and these neurospheres were significantly larger than neurospheres from neural stem cells or olfactory bulbs of p53-wild-type mice. However, neurospheres from p53-null and from p53-wild-type mice possessed similar proportions of neurons, astrocytes and oligodendrocytes, this observation makes an argument against a role of p53 in the differentiation of neural stem cells. In addition, the analyses of the gene expression pattern did not reveal a change in the expression known neuronal differentiation markers (Meletis et al. 2006; Armesilla-Diaz et al. 2009). Also Gil-Perotin and co-workers observed increased proliferation of some, but not all cells in the subventricular

zone of p53-null mice (Gil-Perotin et al. 2006). In contrast, the absence of p53 in stem cells from the olfactory bulb changed the differentiation patterns towards neurogenesis (Armesilla-Diaz et al. 2009). Also, Nagao and co-workers reported that beside a higher self-renewal potential, neural stem cells from p53-null mice produced less astrocytes and more neurons (Nagao et al. 2008), suggesting that p53 function may differ in different tissue stem cells, depending on the organ or area of the organ where the stem cells come from.

Hematopoietic stem cells (HSCs) also appear to be influenced by p53. While a hyperactive mutant of p53 (Tyner et al. 2002) reduced the number of proliferating HSCs the reduction in p53 levels showed he opposite effect (Dumble et al. 2007). In addition, p53 appears to be essential for maintaining HSCs quiescent state since quiescence of HSCs was impaired in the absence of p53 (Liu et al. 2009).

Previous data also demonstrated a major regulatory role of p53 in osteogenic differentiation *in vitro* and in skeletal development and bone remodeling *in vivo* (Lengner et al. 2006; Wang et al. 2006). This activity is most likely du to repression of the two key transcription factors in osteogenic cells, *runx2* and *osterix*, which may directly inhibit the differentiation murine and human mesenchymal stem cells (MSCs) into osteocytic and adipogenic lineages (Molchadsky et al. 2008). The lack of p53, moreover, leads to a higher proliferation rate of bone marrow-derived MSCs, which acquire the typical MSCs surface phenotype earlier than wild type MSCs. Its absence also increases the number of precursors that are able to form colonies and reduces the time that is required for their differentiation into adipocytes or osteocytes. However, the absence of p53 also increased their genomic instability and *c-myc* expression, and enhanced the rate of spontaneous transformation of long-term MSCs cultures (Armesilla-Diaz et al. 2009).

1.2.3.3 P53 and its role in induced pluripotent stem cells

Somatic cells can be reprogrammed into pluripotent stem cells by overexpression of the stem cell factors Oct4, Sox2, Klf4 and c-Myc. These induced pluripotent stem cells (iPS) have a similar behaviour and gene expression profile as native stem cells and are thought to be a potential source of renewable autologous cells, which could

eventually be useful for the treatment of various diseases. Since 2006 have iPS cells been derived from cells of multiple origin, including MEFs, human adult fibroblasts and keratinocytes (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007; Aasen et al. 2008; Lowry, et al. 2008; Nakagawa et al. 2008; Park et al. 2008). However, the low frequency of reprogramming and the tendency to induce malignant transformation of transplanted iPS cells pose doubts about the clinical utility of this approach.

The low reprogramming frequency of transfected implies the existence of a reprogramming barrier. One of the factors that currently discussed as cause of the reprogramming barrier is the p53 tumour suppressor protein. The first evidence for contribution of p53 to the reprogramming barrier came from the observation that downregulation of p53 by siRNA enhanced the reprogramming frequency of human adult fibroblasts significantly, even in the absence of the c-Myc oncogene (Zhao et al. 2008). Then, simultaneously five other reports concerning p53 inhibition of reprogramming were published in *Nature* (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). Kawamura et al. showed that reprogramming factors, as c-Myc, Oct4 and Sox2 induced p53 activity. Reducing of p53 signaling by p53 shRNA or usage *p53*-null MEFs increased efficiency of reprogramming, and re-expressing p53 protein in the *p53*-null MEFs markedly reduced reprogramming efficiency (Kawamura et al. 2009). In another report the authors also demonstrated that *p53*-null MEFs were reprogrammed more efficiently than the wild-type MEFs. Abrogation of p53 in wild-type MEF cells restored reprogramming efficiency to the level as *p53*-null cells. The authors showed that p53 is critically involved in preventing the reprogramming of cells carrying various types of DNA damage, as short telomeres, DNA repair deficiency, as Atm or p53BP1 deficiency, or exogenously inflicted DNA damage (Marion et al. 2009). Hong and co-workers also showed that the loss of p53 function increased the iPS cell induction efficiency in MEFs and in adult human dermal fibroblasts (Hong et al. 2009). They even could generate iPS cells from terminally differentiated T cell, when p53 was inactivated, in contrast with T-cells with functional p53. The authors demonstrated that p21 is important as a p53 target during iPS generation. In general, they concluded that permanent suppression of p53 would lower the quality of iPS and cause genomic instability, therefore, usage of transient suppression of p53 might be useful in generation of iPS for subsequent

medical approaches (Hong et al. 2009). Li et al. studied *Ink4/Arf* locus as a barrier for reprogramming to iPS cells. *Ink4/Arf* locus encodes three tumor suppressors ($p16^{Ink4a}$, $p15^{Ink15b}$ and $p19^{Arf}$) that activate Rb and p53 anti-proliferative pathways (Sharpless, 2005). The authors (Li et al. 2009) found that deficient in *Ink4/Arf* locus and deficient only in *Arf* MEFs were reprogrammed more efficiently, knockdown of both factors had the maximal effect for reprogramming efficiency. *P53*-null and *p21*-null MEFs were reprogrammed more effectively as well, as it was shown in previously described reports (Hong, Takahashi et al. 2009; Kawamura, Suzuki et al. 2009; Marion, Strati et al. 2009). Inhibition both permanent and transient by shRNAs of *Ink4/Arf* locus improved reprogramming efficiency, accelerating the process and increasing the number of successfully reprogrammed cells (Li et al. 2009).

Another study showed that the MEF from early passage numbers produce iPS cells more efficiently that the MEFs from later passages (Utikal et al. 2009). The authors concluded that the senescence is a barrier of reprogramming, similarly to one of the previous studies (Banito et al. 2009). They observed reduced levels of *Arf* and *Inc4* transcripts during reprogramming, this silencing occurs in late intermediate cells. The showed that immortalized cells are reprogrammed more efficiently. MEFs mutant in *p53*, *Ink4/Arf*, or *Arf* showed increased reprogramming efficiency (Utikal et al. 2009).

Thus, one of the tumour suppressing activities of p53 may possibly be the implementation of a reprogramming barrier for somatic cells thus preventing self-renewal and colony formation of mutated cells, and elimination of these mutated cells from the population by apoptosis.

1.3 Aim

P53 is tumour suppressor protein that can activate different cellular programs such as apoptosis, cell cycle arrest and differentiation in response to cellular stress. Moreover, p53 acts as a barrier for reprogramming of somatic cells to iPS and it regulates differentiation at least of some tissue stem cells. According to these novel data about p53 activity in stem cells p53 may be one of the key factors for stem cell fate. In light of these novel activities of p53 in tissue and embryonic stem cells its regulation in these cells becomes more and more important.

The aim of this study was therefore to investigate the regulation of p53 inder normal growth conditions in ES cells, and to study activity of p53 after DNA damage.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and consumables

Name	Source
Acetic Acid	Merck, Darmstadt
Actinomycin D	Sigma Aldrich, Taufkirchen
Agarose	Peqlab, Erlangen
Ampicillin	Roth, Karlsruhe
Ammonium Persulfate (APS)	Roth, Karlsruhe
Agar Agar	Otto Nordwald GmbH, Hamburg
all-*trans*-Retinoic Acid	Sigma Aldrich, Taufkirchen
Bio-Rad Protein Assay	Bio-Rad, München
Bovine Serum Albumine (BSA)	PAA Laboratories GmbH, Pasching
Bovine Donor Serum	Gibco, Invitrogen, Karlsruhe
Bromophenolblue	Roth, Karlsruhe
Coumaric acid	Fluka, Neu Ulm
Cycloheximide	Serva, Heidelberg
Dimethylsulfoxide (DMSO)	Fluka, Neu Ulm
Dithiothreitol (DTT)	Roth, Karlsruhe
6x DNA Loading Buffer	Fermentas, St Leon-Rot, Germany
dNTP Mix Long Range	Peqlab, Erlangen
Draq5	Biostatus Limited, Shepshed, UK
Dulbecco’s Modified Eagle Medium (DMEM)	Gibco, Invitrogen, Karlsruhe
Dullbecco's Modified Eagle Medium - Glutamax	Gibco, Invitrogen, Karlsruhe
Dulbecco’s Modified Eagle Medium High Glucose without L-Methionine and L-Cysteine	Gibco, Invitrogen, Karlsruhe
Ethylenediamine Tetraacetic Acid (EDTA)	Roth, Karlsruhe

EDTA Solution 25 mM	Fermentas, St Leon-Rot, Germany
Ethanol (EtOH)	Roth, Karlsruhe
Ethidium Bromide	Roth, Karlsruhe
Fast Red TR Salt	Sigma Aldrich, Taufkirchen
FBS (Fetal Bovine Serum)	PAA, Pasching, Austria
FBS, ES-cell tested	PAA, Pasching, Austria
Gelatine	Fluka, Neu Ulm
Gene Ruler DNA Ladder Mix	Fermentas, St Leon-Rot, Germany
Gentamicin	Gibco, Invitrogen, Karlsruhe
Glasgow Minimum Essential Medium (GMEM)	Sigma Aldrich, Taufkirchen
Glucose	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
L-Glutamine	Gibco, Invitrogen, Karlsruhe
Goat Serum	Dako, Glostrup, Denmark
Guanidinium-HCL	Sigma Aldrich, Taufkirchen
Hefe extract	Roth, Karlsruhe
Hydrogen Chloride (HCl)	Roth, Karlsruhe
Hydrogen Peroxide	Merck, Darmstadt
Hydromount	National Diagnostics, Atlanta, USA
Igepal CA-630	Sigma Aldrich, Taufkirchen
Imidazole	Sigma Aldrich, Taufkirchen
ImmunoPureR Immobilized Protein A	Pierce, Rockford, USA
Isopropanol	Roth, Karlsruhe
Iscove's Modified Dulbecco' s Medium	Gibco, Invitrogen, Karlsruhe
Leukemia inhibitory factor	PAA Laboratories GmbH, Pasching
Luminol	Fluka, Neu Ulm
Maleic acid	Roth, Karlsruhe
Magnesium Chloride	Invitrogen, Karlsruhe
Magnesium Sulfate	Sigma Aldrich, Taufkirchen
Methanol (MeOH)	Roth, Karlsruhe
β-mercaptoethanol	Roth, Karlsruhe
β-mercaptoethanol	Roth, Karlsruhe
MG132	Sigma Aldrich, Taufkirchen
Milk powder	Saliter, Oberguenzber

Monothioglycerol	Sigma Aldrich, Taufkirchen
Naphtol AS-MX Phosphat	Sigma Aldrich, Taufkirchen
N-ethylmaleimide	Sigma Aldrich, Taufkirchen
Ni^{2+}-nitrilotriacetic acid (NTA)-agarose	Qiagen Hilden, Germany
Non-essential aminoacids	Gibco, Invitrogen, Karlsruhe
Nonident P-40 (NP40)	Roth, Karlsruhe
Nutlin	Calbiochem, San-Diego, USA
Page Ruler Presained Protein Ladder	Fermentas, St Leon-Rot, Germany
Paraformaldehyde	Merck, Darmstadt
Penicillin/streptomycin	Invitrogen, Karlsruhe
1.10-Phenanthroline	Serva, Heidelberg
Phosphate Buffered Saline w/o CaCl2 and MgCl2 1X and 10X	Gibco, Invitrogen, Karlsruhe
PMSF (phenyl methanesulphonyl fluoride)	Sigma Aldrich, Taufkirchen
Potassium Chloride	Roth, Karlsruhe
Proteinase K	Sigma Aldrich, Taufkirchen
QuantiTect Green PCR SYBR	Qiagen, Hilden, Germany
RiboBlockTM RNAse Inhibitor	Fermentas, St Leon-Rot, Germany
Rotiphorese® Gel30: Acrylamide/ bis-acrylamide (30%/0,8%)	Roth, Karlsruhe
Rotisol	Roth, Karlsruhe
Sodium Acetate	Roth, Karlsruhe
Sodium Chloride	Roth, Karlsruhe
Sodium Dodecyl Sulphate (SDS)	Roth, Karlsruhe
Sodium Hydroxide	Roth, Karlsruhe
Sodium Hydrogen Phosphat	Roth, Karlsruhe
Sodium Dihydrophosphat	Roth, Karlsruhe
Tetramethyl ethylen diamine (TEMED)	Roth, Karlsruhe
Tris-base	Roth, Karlsruhe
Trypsin type XI: from bovine pancreas	Sigma Aldrich, Taufkirchen
Trypton/Pepton	Roth, Karlsruhe
Triton-X-100	Roth, Karlsruhe
Tween 20	Sigma Aldrich, Taufkirchen
Urea	Roth, Karlsruhe

2.1.2 Bacteria and eukariotic cell lines

2.1.2.1 Bacteria

Name	Genotype
E. Coli DH5 α	F-, nd A1, hsd R17 (rk-, mk+), sup E44, thi-1, λ-, rec A1, gyr A96, relA1

2.1.2.2 Eukaryotic cell lines

All the cells grew adherently in monolayers at 37 °C with 7% CO_2 on Cellstar® Petri dishes and cell culture plates (Greiner Bio-one, Frickenhausen, Germany).

Name	Source and description
R1	Mouse Embryonic Stem cell Line, derived from male blastocyst, hybrid of two 129 substrains (129X1/SvJ and 129S1/SV-+p+Tyr-cKitlSl-J/+), provided by Dr. A Rolletschek
D3	Mouse Embryonic Stem Cell Line, derived from blastocysts of a 129S2/SvPas mouse, provided by Dr. A Rolletschek
CGR8	Mouse Embryonic Stem Cell Line, derived from blastocyst of mouse embryo, strain 129P2/OlaHsd, provided by Dr. A Rolletschek
NIH3T3	Mouse Fibroblast Cell Line, obtained from the European Cell Culture Collection (ECCC)
MEF	Mouse Embryonic Fibroblast Cells, derived from mouse embryo 13.5 days, C57BL/6 strain

2.1.3 Oligonucleotides

2.1.3.1 Primers for cloning

Specific oligonucleotides were used to amplify genes of interest by Polymerase Chain Reaction. The oligonucleotides contained specific and unique restriction sites to allow cloning of the PCR fragments into a suitable vector.

Name	Sequence (restriction site underlined)	Retriction site
125-a-fo	TCGAGGATCCTCCTTTCTATCTTCTGGGGC	BamH1
125-a-re	TCGAGCTAGCGTGATCAGAGATTGAGTTTC	Nhe1
125-b-fo	TCGAGGATCCGTGTTTCAATTAGTATTTAG	BamH1
125-b-re	TCGAGCTAGCCGAAAACCATTGTTCTTTGCG	Nhe1

2.1.3.2 Sequences of siRNAs

Name	Sequence
Hausp siRNA	GCAGUGCUGAAGAUAAUAATT
PirH2 siRNA	AUUUAUGCCUAACCACGAATT
L26 siRNA	GAGCUGAGACAGAGAAGUA
Ubc13 siRNA	CCAGAUGAUCCAUUAGC
ctrl siRNA	AACCCCUUUUAAAAGGGG

2.1.3.3 Primers for RT-PCR

Name	Sequence
mdm2-fo	TGGAGTCCCGAGTTTCTCTG
mdm2-re	AGCCACTAAATTTCTGTAGATCAT
noxa-fo	CGTCGGAACGCGGCCAGTGAACCC
noxa-re	TCCTTCCTGGGGAGGTCCCTTCTTGC
p21-fo	CCAGGCCAAGATGGTGTCTT
p21-re	TGAGAAAGGATCAGCCATTGC
puma-fo	ACCCCATCGCCTCCTTTCTCCG
puma-re	ATACAGCGGAGGGCATCAGGCG
p53-fo	CCTCATCCTCCTCCTTCCCAGCAG
p53-re	AACAGATCGTCCATGCAGTGAGGTC
RibPO(34B4)-fo	GAAGGCTGTGGTGCTGATGG
RibPO(34B4)-re	CCGGATATGAGGCAGCAG

2.1.3.4 Primers for the determination of microRNA-125a and 125b expression

Name	Sequence
RT-125a	CGCGCCCTCCCTGAGACCCTT
125a-fo	GTCGTTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACCACAGG
RT-125b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ATCACAA
125b-fo	GTGGCACACCCTGAGACCCTAAC
Universal -reverse	GTGCAGGGTCCGAGGT

2.1.4 Primary Antibodies

WB= western blot; IF= immunofluorescence; PBS= Phosphat buffered saline; BSA= bovine serum albumine; RT= room temperature; ON= overnight

Name	Description	Experimental conditions	Produ-cer	Applica-tion
Anti-p53 (Pab 421)	Mouse monoclonal antibody recognizing p53 of mouse and human origin	Used as supernatant from hybridoma cells, ON 4ºC or 4 h RT	Calbio-chem	WB, IF, IP
Anti-p53 (CM-5)	Rabbit polyclonal antibody recognizing murine p53	Diluted 1:5000 in PBS/0.02% Tween 20/5% milk powder, 1 h RT	Vector Labora-tories	WB
		Diluted 1:500 in blocking buffer (1% goat serum, 1% BSA in PBS), 1 h RT		IF
Anti-Mdm2 (4B2)	Mouse moonoclonal antibody recognizing Mdm2 of mouse and human origin	Used as a supernatant from hybridoma cells, ON 4ºC	Calbio-chem	WB, IF, IP

Anti-HAUSP (H-200)	Rabbit polyclonal antibody recognizing HAUSP of human, mouse origin	Diluted 1:5000 in PBS/0.02% Tween 20/5% milk powder, 2 h RT	Santa-Cruz Biotech	WB
		Diluted 1:200 in blocking buffer (1% goat serum, 1% BSA in PBS), 1 h RT		IF
Anti-GRP75 (H-155)	Rabbit polyclonal antibody recognizing GRP75 of rat, mouse and human origin	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Santa Cruz Biotech.	WB
		Diluted 1:250 in blocking buffer (4% goat serum in PBS), 1 h RT		IF
Anti-L26	Rabbit polyclonal antibody recognizing L26 protein	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Cell Signal-ling	WB
Anti-Ubc13	Rabbit polyclonal antibody recognizing Ubc13	Diluted 1:2000 in PBS/0.02% Tween 20/5% milk powder, 2h RT	Acris Antibo-dies	WB
		Diluted 1:250 in blocking buffer (1% goat serum, 1% BSA in PBS), 1 h RT		IF
Anti-p21	Rabbit polyclonal antibody recognizing p21	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Santa Cruz Biotech.	WB
Anti-Puma	Rabbit polyclonal antibody recognizing Puma	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Cell Signal-ling	WB
Anti-Noxa	Goat polyclonal antibody recognizing Noxa	Diluted 1:500 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Santa Cruz Biotech.	WB
Anti-Oct3/4	Mouse monoclonal antibody	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, 2 h RT	Santa Cruz Biotech.	WB

	recognizing Oct 3/4			
Anti-Nanog	Rabbit polyclonal antibody recognizing Nanog	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, 2 h RT	Milipore	WB
Anti-Parc	Mouse monoclonal antibody recognizing Parc	Diluted 1:2000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Biole-gend	WB
		Diluted 1:250 in blocking buffer (1% goat serum, 1% BSA in PBS), 1 h RT		IF
Anti-K63 (FK2)	Mouse monoclonal antibody recognizing proteins linked with ubiquitin via Lys 63	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, 2 h RT	Enzo Life Sciences	WB
Anti-mono- and poly-ubiquitinated proteins (Fk1)	Mouse monoclonal antibody recognizing ubiquitinated proteins	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, 2 h RT	Enzo Life Sciences	WB
Anti-PirH2 (T-18)	Goat polyclonal antibody recognizing PirH2	Diluted 1:500 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Santa Cruz Biotech	WB
Anti-Foxo3A (H-144)	Rabit polyclonal antibody recognizing Foxo3A	Diluted 1:1000 in PBS/0.02% Tween 20/5% milk powder, ON 4ºC	Santa Cruz Biotech	WB
		Diluted 1:250 in blocking buffer (1% goat serum, 1% BSA in PBS), 1 h RT		IF

2.1.5 Secondary antibodies

All secondary antibodies rabbit anti-goat, goat anti-mouse, goat anti-rabbit HRP-conjugated were purchased from DAKO Diagnostic GmBH (Glostrup, Denmark). All secondary Alexa Flour 546 goat anti-rabbit and goat anti-mouse, Alexa Fluor 488 goat

anti-mouse and goat anti-rabbit antibodies were purchased from Invitrogen (Karlsruhe, Germany).

2.1.6 Enzymes

All enzymes (restriction enzymes NheI and BamHI, DNAse I, RevertAid^{TM} H MinusM-MuLV reverse transcriptase (MLVRT), Pfu Polymerase, Dream Taq Polymerase, T4 DNA Ligase, Fast Alkaline Phosphatase (FastAP) were purchased from Fermentas (St Leon-Rot, Germany).

2.1.7 Plasmids

The following plasmids were used for transfection of embryonic stem cells:

Name	Description
pcDNA3.1-Hausp	Expression plasmid containing the cDNA of HAUSP, carrying a V5-tag, provided by Wei Gu.
pcNA3.1-Mdm2	Expression plasmid containing the cDNA of Mdm2, provided by Arnold Levine.
His-Ubi	Expression plasmid containing the cDNA of ubiquitin carrying a 6x His-tag, provided by Sibylle Mittnacht.
1816-L26	1816 expression plasmid containing the cDNA of L26, carrying a Flag-tag, provided by Moshe Oren
miRNASelectTM pEGFP-miRNA-125a	Expression plasmid containing murine miRNA-125a and GFP.
miRNASelectTM pEGFP-miRNA-125b	Expression plasmid containing murine miRNA-125b and GFP.
miRNASelectTM pEGFP-miRNA-Null	Vector for micro-RNA expression containing GFP.

2.2 Methods

2.2.1 Nucleic Acid Techniques

2.2.1.1 Transformation of bacteria

For transformation typically 50 µl of chemically competent bacteria DH5α were incubated for 20 min on ice with 10 µl of a ligation reaction or with 1 µl of a purified plasmid. Thereafter the cells were heat-shocked at 37ºC for 5 min or at 42ºC for 2 min and incubated on ice for 2 min. The transformed bacteria were incubated in a final volume of 1 ml of LB (1xLB is 10 g of Trypton, 5 g of Hefe extract, 10 g of sodium chloride for 1 L of medium) for 30 min at 37ºC on a shaker. In the meantime an agar plate (1.5% agar agar in LB medium) was warmed to RT. Finally 100 µl of the bacterial suspension were spread over the agar plate and incubated ON at 37ºC. In case of ligase reaction mix all bacterial suspension was spread over the agar plate.

2.2.1.2 Small-scale purification of plasmid DNA

For mini-preparation of DNA, 3 ml of transformed bacteria were incubated in LB with ampicillin (50 µg/ml) at 37ºC ON with constant shaking at 220 rpm. Thereafter 1 ml of the bacteria culture was transferred into a 1.5ml-reaction tube and centrifuged at 13000 rpm for 30 s at 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17). Cells were washed once with ice-cold TE buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0). The SNT was removed and the pellet was resuspended in 200 µl of solution P1 of the Plasmid Maxi Kit® (Qiagen, Hilden, Germany) supplemented with 400 mg/ml RNase A followed by incubation for 5 min at RT. Alkaline lysis was performed by addition of 200 µl of solution P2 of the Plasmid Maxi Kit® and. The solution was then incubated for 5 min on RT. Thereafter the suspension was neutralized by addition of 200 µl of buffer P3 from the Plasmid Maxi Kit® followed by vortexing and incubation for 5 min on ice. The solution was then centrifuged at 13000 rpm for 15 min at 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17) to separate the lysate from the cells debris and the SNT was transferred into a new reaction tube. If necessary the centrifugation was

repeated to enable complete removal of the debris. The DNA was precipitated by addition of 1 ml of ice-cold ethanol to 400 µl of the SNT and incubation for 30 min at -80ºC. The DNA was collected by centrifugation at 13000 rpm for 15 min at 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17), the SNT was discarded and the DNA pellet was washed from residual salts with 200 µl of ethanol (80%). After centrifugation at 10000 rpm for 2 min at 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17), the SNT was discarded and the residual ethanol was allowed to evaporate. The DNA was resuspended in 30 µl of TE buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0).

2.2.1.3 Large-scale purification of plasmid DNA

For large-scale purification of plasmid DNA, a Plasmid Maxi kit (Qiagen, Hilden - Germany) was used. Bacteria were cultured in 300 ml of LB containing ampicillin (50 µg/ml – f.c.) at 37ºC ON with constant shaking at 220 rpm. The next day, bacteria were collected by centrifugation at 5000 rpm for 20 min at 4ºC (Beckmann J2-HS centrifuge, rotor JA-10) and resuspended in 10 ml of buffer P1 containing 400 µg/ml RNase A. After incubation for 10 min at RT alkaline lysis was performed by addition of 10 ml of buffer P2. The solution was mixed gently and the reaction was allowed to proceed for 10 min at RT. Thereafter the solution was neutralized by addition of 10 ml of buffer P3 and the whole mixture was mixed vigorously. To separate the lysate from the debris the suspension was centrifuged at 4000 rpm for 15 min at 4ºC (Heraeus, Biofuge PrimoR). After centrifugation, the SNT was loaded into a Tip 500 column (Qiagen, Hilden, Germany) that had been equilibrated with 15 ml of buffer QBT (Qiagen, Hilden, Germany). The column was washed twice with 30 ml of buffer QC (Qiagen, Hilden, Germany) and the DNA was eluted with 15 ml of buffer QF (Qiagen, Hilden, Germany). The eluted DNA was precipitated by addition of 10 ml of isopropanol and incubation for 15 min on ice. The precipitated DNA was collected by centrifugation of the solution at 9000 rpm for 15 min at 4ºC (Beckmann Avanti J-20). The SNT was discarded and the pellet was resuspended in 5 ml of ethanol (80%). After centrifugation at 9000 rpm for 5 min at 4ºC (Beckmann Avanti J-20) the SNT was carefully discarded

and the pellet was dried at 50ºC. The DNA was dissolved in 200-300 µl of TE buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0).

2.2.1.4 Determination of the plasmid DNA concentration

To determine the concentration of DNA, the optical density (OD) of a DNA solution was measured at 260, 280 and 230 nm using the NanoDrop® device and the software ND-1000 (version 3.1.2). An OD_{260} of 1 corresponds to 50 µg/ml of double-stranded DNA. A ratio of OD_{260}/OD_{280} of 1.8 indicates a nucleic acid preparation that is relatively free of protein contamination. A ratio of OD_{260}/OD_{230} above 1.6 indicates a solution of DNA that is free of organic chemicals and solvents.

2.2.1.5 Separation of nucleic acids by agarose gel electrophoresis

DNA was separated according to its size by agarose gel electrophoresis using a horizontal gel chamber and agarose concentrations ranging from 0.8 to 2%, depending on the size of the fragments to be separated. The respective amount of agarose was dissolved in TAE buffer (0.04 M Tris pH 7.2, 0.02 sodium acetate, 1mM EDTA) and dissolved by boiling. Thereafter, the agarose solution was cooled down to about 40ºC. Ethidium bromide was added to a final concentration of 0.4 µg/ml and the solution was poured into a horizontal gel chamber. A comb was placed into the agarose solution to allow the formation of slots where samples could be placed. After polymerisation, the chamber was filled with TAE buffer. The samples were mixed with 6x DNA Loading Buffer (Fermentas) and loaded onto the gel. Electrophoresis was carried out at 80-120 V and the separation of the DNA was visualized under UV light.

2.2.1.6 Extraction of DNA from agarose gels.

For the extraction of DNA from agarose gels, the PeqGold Gel extraction Kit (PeqLab, Erlangen) was used according to the manufacturer's recommendation. After separation by gel electrophoresis, DNA was visualized under UV light and gel pieces containing the DNA of interest were cut out, weighted and transferred into a 1.5ml reaction tube. To 100 µg of the DNA/agarose, 100 µl of Binding Buffer were added and the solution was incubated at 60°C for 10 min with vortexing each 2-3 min to dissolve the agarose. Once the agarose had been dissolved, the content of the tube was transferred to a purification column connected to a collection tube and centrifuged for 1 min at 13000 rpm (Thermo Scientific centrifuge, Heraues Fresco 17). The flow-through in the collecting tube was discarded and the column was washed once with 300 µl Binding Buffer and twice with 500 µl Washing Buffer. The column was spun to remove any remaining ethanol from the Washing Buffer and the DNA was eluted with 30 µl Elution Buffer.

2.2.1.7 Extraction of RNA from eukaryotic cells

For the preparation of total RNA, the RNeasy kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendation. Briefly, $5x10^5$ cells were harvested, washed once with ice-cold PBS, and resuspended in 300 µl of RLT Lysis Buffer (Qiagen, Hilden, Germany). Cells were disrupted and homogenized by processing five times with 20-gauge needle syringe. Then, 300 µl of 70% ethanol was added, containment of the tube was properly mixed and loaded to column (maximum volume is 700 µl). Column was spun 10000 rpm for 20 sec (Thermo Scientific centrifuge, Heraues Fresco 17), and flow-through was discarded. Column was sequentially washed by 400 µl of RW1 buffer, twice with 500 µl of RPE buffer. To remove rests of ethanol empty column was spun once more for 1 min 13000 rpm (Thermo Scientific centrifuge, Heraues Fresco 17). RNA was eluted with 30µl of RNAse-free water (Qiagen). To determine the concentration of the RNA solution, the optical density (OD) was determined at 260, 280 and 230 nm using the NanoDrop®

device and the software ND-1000 (version 3.1.2). An OD_{260} of 1 corresponded to 40 μg/ml of single stranded RNA.

2.2.1.8 cDNA synthesis

150 ng – 1 μg RNA were treated with DNase I (Fermentas) in the presense of the RiboBlock^{TM} RNAse inhibitor (Fermentas) (1 μl for 10 μl of reaction mix) to remove residual genomic DNA. Thereafter, DNAse activity was inhibited by the addition of 1 μl of 25 mM EDTA and incubation of the samples at 65°C for 10 min. RNA was transcribed into cDNA using random primers (Invitrogen) and RevertAid^{TM} H MinusM-MuLV reverse transcriptase (MLVRT) (Fermentas). First, RNA was incubated with 1 μl of 200 ng/μl Random primers 70°C for 5 min. Then, 10 μl of Master Mix (2 μl of 10 mM dNTP mix, 1 μl of MLVRT (Fermentas), 4 μl of 5x reaction buffer, 4 μl of water) was added to RNA. For cDNA synthesis next program was used: 10 min for 25°C, 60 min for 42°C, 10 min for 70°C, then 4°C. cDNAs were stored at -20°C. Before to use, cDNAs were diluted 5 times with bi-distilled water.

Quality of cDNA was checked by PCR using primers for RibPO (34B4) gene. 20 μl of PCR mix contained 4 μl of cDNA, 0.4 μM (f.c.) of forward and reverse primers, 0.2 mM (f.c.) of dNTP Mix, 1.5 mM (f.c.) of $MgSO_4$, 2 μl of 10x Taq polymerase buffer, 0.5 μl of Dream Tag Polymerase (Fermentas). PCR program was 95°C for 3 min, then 30 cycles of 95°C for 30 sec, 60°C for 1 min, 72°C for 1 min, then 72°C for 10 min using Thermocycler GeneAmp PCR System 2400 (Perkin Elmer). Products of PCR were analysed by separation in agarose gel.

2.2.1.9 qRT-PCR

Real-time PCR was performed in duplicates with a Quanti Tect Green PCR SYBR (Qiagen) using primers specific for genes of interest. For each run, in addition to genes of interest RT-PCR using primers for RibPO (34B4) gene was performed to normalize signals of other genes to the signals of RibPO expression. For 20 μl of reaction 10 μl of

2xSYBR Green, 10 µM of forward primer and reverse primer and 4 µl of cDNA were used. RT-PCR was performed in ABgene^R PCR Plates (Thermo Scientific, UK). 7000 ABI sequence detection system was used to perform RT-PCR using next program:

Holding Stage:

15 sec 95°C

Cycling Stage (40x):

1: 15 sec 95°C (denaturation)

2: 30 sec 60°C (annealing and extension)

Melt Curve Stage:

1: 15 sec 95°C

2: 1 min 60°C

3: 15 sec 95°C

Analysis of RT-PCR was carried out using software for 7000 ABI sequence detection system.

2.2.1.10 cDNA synthesis of micro-RNAs

Micro-RNAs were extracted (together with total RNA) using the RNeasy kit (Qiagen) according to the manufacturer's recommendation. cDNA synthesis was carried out according to (Varkonyi-Gasic et al. 2007). 500 ng of RNA in a volume of 10 µl were mixed with 1 µl of a Stem-Loop primer (1µM) specific for each micro-RNA and 1 µl of 10 mM dNTP Mix, and incubated at 65°C for 5 min. Thereafter, 4 µl of RevertAid^{TM} H MinusM-MuLV reverse transcriptase buffer (5x; Fermentas), 1 µl DTT (1M), 2 µl of water and 1 µl RevertAid^{TM} H MinusM-MuLV reverse transcriptase (Fermentas) were added and cDNA synthesis was performed for 30 min at 16°C. Thereafter, 60 cycles with 30 sec at 25°C, 1 sec at 50°C, and then 10 min at 85°C were performed in Thermocycler GeneAmp PCR System 2400 (Perkin Elmer).

2.2.1.11 PCR for the determination of microRNA expression

Expression of microRNA-125a and 125-b was tested by PCR of cDNA synthesis products. 20 μl of PCR reaction contained 4 μl of cDNA, 0.4 μM of forward primer specific for each microRNA, 0.4 μM of universal reverse primer, 2 μl of 2 mM dNTP Mix, 1.5 mM of $MgSO_4$, 2 ml of 10x Dream Taq Polymerase buffer, 0.5 ml of Dream Tag Polymerase (Fermentas). PCR profile was 2 min for 95°C, 18 cycles for 30 sec 95°C, 1 min 58°C, 1 min 72°C, thereafter 7 min for 72°C using a thermocycler GeneAmp PCR System 2400 (Perkin Elmer). After reaction PCR products were separated by agarose gel.

To normalize signals for microRNA expression, general cDNA synthesis (as 2.2.1.8) was performed from the 500 ng of the same sample of RNA and PCR for RibPO probe was carried out. Products of reaction were analysed after separation in agarose gel under UV light.

2.2.1.12 Cloning of microRNA-125a and 125b

2.2.1.12.1 PCR

For cloning of miRNA-125a and 125b, microRNAs were amplified by PCR using mouse genomic DNA as a template. Murine miRNA-125a and miRNA-125b are encoded by genes located on chromosomes 17 and 9 and 11, respectively, as 100 base pair stem-loop precursors. PCR primers were designed to get PCR product containing miRNA precursor flanked on both sides by its 100 base pair native intron sequences. This provides that the vector expresses the miRNA precursor in its native context while preserving the putative hairpin structure to ensure biologically relevant interactions with the endogenous processing machinery and regulatory partners, which is required for the correct cleavage of microRNA. Forward and reverse primers contained also sites for restriction endonucleases NheI and BamHI, respectively.

Mouse genomic DNA was extracted from mouse embryonic fibroblasts. 10^6 cells were resuspended in 300 μl of Lysis buffer (50 mM KCl, 10 mM tris, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 0.1 mg/ml Proteinase K)

and incubated overnight for 55°C. Then, the lysate was incubated for 30 min at 100°C. 1 μl of lysates was used for PCR.

PCR was carried using a thermocycler GeneAmp PCR System 2400 (Perkin Elmer). The PCR mix contained 1 μl of genomic DNA, 1 U of Pfu proof-reading polymerase (Fermentas), 0.2 mM dNTP Mix (f.c), 0.4 μM of forward primer (f.c.), 0.4 μM of reverse primer (f.c.), 1.5 mM (f.c.) of $MgSO_4$, 4 μl of 10x Pfu proof-reading DNA polymerase buffer in volume of 40 μl. The PCR program was: 95°C for 2 min, 30 cycles of 95°C 30 sec, 58°C for 1 min, 72°C for 1 min, thereafter 5 min at 72°C.

2.2.1.12.2. Restriction digestion

The resulting PCR products containing microRNA genes were extracted from agarose gel after separation (see 2.2.1.5), digested by NheI and BamHI and cloned into the miRNASelect^{TM}pEGFP-mir vector (Cell Biolabs) using NheI and BamHI restriction sites.

Digestion was performed using Fast Digest NheI and BamHI restriction enzymes (Fermentas) at 37°C for 2 h. Reaction was stopped by incubation at 65°C for 15 min.

miRNASelectTMpEGFP-mir vector after digestion by NheI and BamHI was treated with 1 U of Alkaline Phosphatase (FastAP, Fermentas) for 15 min at 37°C, reaction was stopped incubation at 65°C for 10 min.

After digestion and treatment by FastAP DNA fragments were separated in agarose gel. Pieces of gel containing DNA of interest were cut and DNA was extracted (see 2.2.1.5).

2.2.1.12.3 Ligation of DNA fragments

All ligations were performed using 5 U of the enzyme T4 DNA ligase (Fermentas, St Leon-Rot - Germany). For insertion of a specific DNA fragment into a vector the ratio insert:vector was usually 1:3 or 1:6. The reaction was carried in presence of T4 DNA ligase buffer (containing a final concentration of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA) in a final volume of 30 μl for 1 hour at 22ºC or for 4 hours at 16ºC or ON at 4ºC. Every ligation experiment was

performed incubating the empty vector digested with the same restriction enzymes as control. The reaction was stopped by inactivation of the enzyme at 65ºC for 10 min.

2.2.2 Cell culture and transfection methods

2.2.2.1 Culturing of eukaryotic cells

All mammalian cells were cultured under standard conditions at 37ºC, 7% of CO_2 and 95% of humidity in a cell culture incubator (Forma Scientific Labortech GmbH, Gottingen, Germany). All cells were grown in sterile Cellstar® Petri dishes (Greiner Bio-One, Frickenhausen, Germany) of different sizes depending on the experimental conditions.

R1 and D3 ES cells were cultured in DMEM - GlutaMAX™-I medium (Invitrogen) supplemented with 15% fetal bovine serum (FBS; PAA), 0.1 mM β-mercaptoethanol, 1% penicillin/ streptomycin (Invitrogen) and 1000 units/ml LIF. R1 and D3 cells were cultured on feeder cells (see 2.2.2.3). Where indicated, ES cells were cultured on dishes or coverslips coated with 0.1% gelatine for up to two passages.

CGR8 ES cells were grown in Glasgow Minimum Essential Medium (Sigma) supplemented with 10% fetal bovine serum, 40 µg/ml gentamycin, 100 units/ml LIF, 50 µM b-mercaptoethanol (Gibco), 2 mM L-Glutamine (Invitrogen) and 1 mM non-essential amino acids (Invitrogen) on dishes that had been coated with 0.2% gelatine.

All ES cell lines were sub-cultured every second day.

Trypsin for ES culture contained 0.5 g of Trypsin Type XI (Sigma), 8 g of NaCl, 0.4 g of KCl, 1 g of Glucose, 0.589 g of $NaHCO_3$, 0.29 g of EDTA for 1 L.

NIT3T3 cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% donor bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen). Mouse embryonic fibroblasts were cultured in DMEM - GlutaMAX™-I medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAA) and 1% penicillin/streptomycin (Invitrogen) and used between passage 0 and 5.

2.2.2.2 Freezing and thawing of the cells

For freezing cell were harvested and resuspended in freezing medium (10% of DMSO in corresponding for each cell line medium), and divided on aliquots ($5x10^5$ cells per Cryotube (Nunc). Cells in Cryotubes were inserted in Nalgene Cryo 1°C Freezing Container to -80°C, thus providing rate of temperature decrease 1°C per 1 min. Next day, tubes with the cells were transferred to liquid nitrogen.

To thaw cells, cryotubes were transferred to water bath at 37°C for several minutes. As soon as cell suspension was thawed, tube containment was transferred to 15 ml Falcon tube containing 10 ml of corresponding for each cell line medium. The cells were centrifuged 1000 rpm 2 min (Heraeus Megafuge 1.0), resuspended in fresh medium and plated to the cell culture dish.

2.2.2.3 Preparation of plates to culture ES cells

Mouse embryonal fibroblasts (passages 0 to 5) that had been irradiated with 6.3 Gray, served as feeder cells. For each 6 cm dish $9.3x10^5$ feeder cells were seeded. Each second day medium for feeder cells was changed. These plates were used no longer than 10 days.

For gelatin-coating cell-culture dishes with or without coverslips were incubated for 1 h with 0,1% or 0,2% gelatin on PBS solution at RT. Then gelatine was discarded, plates were air-dried and ready to use. Gelatine-coated plates can be stored during 10 days at 4°C.

2.2.2.4 Transfection of ES cells

R1 and D3 cells were transfected either using Effectene™ Transfection Reagent (Qiagen) or the mouse ES cell Nucleofector Kit (Amaxa, Lonza Cologne) according to manufacturer's recommendations. For transfection with the ES cell Nucleofector Kit, 10^6 cells were resuspended in Amaxa buffer. 6 µg of plasmid DNA (for overexpression) or 80 nM siRNA (for knockdown) were added to the mixture. The whole sample was

transferred into an electroporation cuvette (Amaxa, Lonza Cologne) and electroporated using the program A23 or A24 of the electroporation device (Amaxa). Thereafter, cells were resuspended in medium and plated onto gelatine-coated plates and analysed at 24 hours after transfection.

For transfection with Effectene™ Transfection Reagent (Qiagen), $5x10^5$ cells were seeded in gelatine-coated 6-well plates. After cells have attached to the dish (5 hours or overnight), 1 μg of plasmid DNA or 100 nM of siRNA were mixed with 100 μl of EC buffer (Qiagen) and 3.2 μl of Enhancer (Quiagen). The mix was vortexed for 1s and incubated for 10 min at RT. Thereafter, 8 μl of Effectene™ (Qiagen) were added to the mixture. The samples were vortexed for 5 s and incubated for 15 min at RT. During this incubation time the plated ES cells were washed with PBS and 1.6 ml of fresh medium was added. After the 15 min incubation time, 600 μl of fresh medium were added to the Effectene-DNA/siRNA mixtures, the samples were mixed and transferred onto the plated cells. Cells were analysed at 24 h or 48 h post-transfection.

2.2.2.5 Differentiation of ES cells

2.2.2.5.1 Differentiation of ES cells by all-*trans-retinoic acid*

R1 or D3 cells were trypsinized and plated into cell culture dishes that had been coated with 0.1% gelatine in GlutaMAX™-I medium (Invitrogen) supplemented with 10% donor bovine serum (Gibco), 1% penicillin/streptomycin (Invitrogen), 0.1 mM of β-mercaptoethanol and all-*trans*-retinoic acid (f.c. 1 μM) (Sigma) for 6 to 12 days.

2.2.2.5.2 Differentiation of ES cells by formation of embryoid bodies

R1 or D3 ES cells were trypsinized and counted. 400 cells/drop were cultured in hanging drops at the lid of a Petri dish in IMDM-medium (Invitrogen) supplemented with 20% FBS (PAA), 1% penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM non-essential amino acids (Invitrogen) and 150 mM monothyoglycerol for 2 days. Thereafter, aggregates were collected and cultured in suspension for additional 2 days before the embryoid bodies (EBs) were transferred into

a cell culture dish (15-20 per 6 cm dish) that had been coated with 0.1% gelatine. At this step, the culture medium of the EBs was supplemented with 1 μM of all-*trans*-retinoic acid (Sigma) and the cells were cultured for additional 4 days (EB-4d cells). EB-4d cells from one plate were trypsinized and plated onto three plates coated with 0.1% gelatine and cultured for additional 4 - 5 days where indicated.

2.2.2.6 Irradiation of cells and special treatments

Cells were irradiated using a cobalt-γ-source at a dose rate 1 Gy per minute.

Nutlin-3 (Calbiochem) was used at a final concentration of 10 μM, MG132 (Calbiochem) was used at a final concentration of 5 μM, cycloheximide (Sigma) was used at a final concentration of 50 μM and actinomycin D (Sigma) was used at a final concentration of 5 μg/ml.

2.2.2.7 Alkaline Phosphatase Staining

Cells were washed twice by PBS and fixed by 2 ml of 4% paraformaldehyde solution for 20 min at RT. Afterwards, cells were washed three times with tris-maleat buffer (1 M maleic acid, 0,02 M tris, pH 9.0) for 10 min at 4°C. Alkaline phosphatase activity was developed by incubation for 20 min in reaction solution at RT (0.1 % Fast Red TR Salt, 0.02% Naphtol AS-MX Phosphate, 0.08 % $MgCl_2$ in tris-maleat buffer). Reaction solution was exchanged to PBS and developing of red color (alkaline phosphatase - positive staining) was investigated under light microscope.

2.2.3 Protein Methods

2.2.3.1 Determination of protein localisation by immunofluorescence microscopy

For immunofluorescence microscopy, glass coverslips were coated with 0.1% gelatin. Thereafter cells were plated onto the gelatin-coated cover slips and incubated for 24 hours. The following day, the medium was aspirated, the cells were washed once with PBS and fixed with 500 µl of methanol-acetone (1:1) on ice for 8 min. The methanol-acetone mix was aspirated and cells were washed three times with PBS before they were incubated for 30 min at room temperature with blocking solution (1% goat serum, 1% BSA in PBS). After blocking, the respective primary antibody diluted in blocking buffer was added. Samples were incubated for 1 hour at room temperature or ON at 4°C. After washing twice with PBS, secondary antibodies diluted 1:1000 in blocking buffer, were applied together with Draq5, diluted 1:1000 in blocking buffer. The samples were incubated for 30 min at room temperature, washed three times with PBS and the cover slips were mounted onto microscope slides using Hydromount (National Diagnostics, Atlanta, USA) as a mounting medium. Cells were analysed using the Zeiss LSM510 confocal microscope and the LSM LSell5 Image Examiner software.

2.2.3.2 Preparation of protein lysates from cells

For protein extraction, cells in a 6 cm cell culture dish were typically washed with 5 ml of ice-cold PBS and scraped in 1 ml PBS. The cell suspension was transferred into a 1.5ml reaction tube and cells were collected by centrifugation for 5 min at 4000 rpm and 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17). The SNT was discarded and cells were resuspended in 100 µl of lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% NP40, 1 mM of PMSF) for 15 min and then centrifuged for 15 min at 13000 rpm and 4ºC (Thermo Scientific centrifuge, Heraues Fresco 17). SNT was transferred to a new tube and was used for analysis.

2.2.3.3 Determination of the protein concentration of cellular lysates

For quantifying the protein concentration of cellular lysates, the Bradford assay was used. Typically 2 µl of protein extract were diluted in 1 ml of 1X Bio-Rad Protein Assay solution (Bio-Rad, Munich, Germany). To determine the intensity of the

background, 2 µl lysis buffer were diluted with 1 ml of Bradford solution 200 µl of the samples were pipetted into a 96 well plate and the intensity of the signal was defined at 595 nm using the EL_X 808 UI Ultra Microplate Reader (software KC4 version 3.01). Serial dilutions of bovine serum albumine (BSA) (2 µg – 20 µg) were used to plot standard curve using defined amounts of BSA and to calculate the final protein content using software KC4.

2.2.3.4 Separation of proteins by SDS PAGE

The Bio-Rad apparatus for mini-gels (Bio-Rad) was used to cast and run SDS-PAGE (polyacrylamide gelelectrophoresis) gels. Typically gels containing 10% polyacrylamide were used for the separation of cellular lysates (for 20 ml of a 10% gel solution 7.9 ml double distilled water, 6.7 ml 30% Acrylamide mix, 5 ml 1.5 M Tris pH 8.8, 200 µl 10% SDS, 200µl APS and 8 µl TEMED were used). The separation gel was poured between two glass plates (a longer and a shorter one) that were separated by the spacers and that had been inserted into a casting stand. The space between the glass plates was filled with acrylamide solution up to two cm from the top of the shorter glass plate and the acrylamide solution was overlaid with isopropanol. After polymerization of the separation gel, the isopropanol was discarded and the top of the gel was washed with distilled water. Thereafter, the stacking gel (for 10 ml of solution 6.8 ml double distilled water were mixed with 1.7 ml 30% acrylamide mix, 1.25 ml 1 M Tris pH 6.8, 100 µl 10% SDS, 100 µl APS and 10 µl TEMED) was poured on top of the separation gel. A comb was inserted to allow the formation of slots where the samples could be placed. After polymerisation of the stacking gel, the gel was removed from the casting stand and inserted into a running chamber that was filled with SDS-PAGE running buffer (0.193 M Glycin, 0.043 M Tris base, 0.2% SDS). The desired amount of protein (usually 30 – 50 µg) was diluted 1:1 with 2x sample buffer (4% sodium dodecyl sulphate, 0.16 M Tris, pH 6.8, 20 % glycerol, 4% β-mercaptoethanol, 0.002% bromphenol blue), the samples were heat-denatured for 5 min at 95°C and pipetted into the slots of the stacking gel. Electrophoresis was performed at 100V for 3-4 hours until the bromphenol blue front reached the bottom of the glass plates.

2.2.3.5 Western blotting and protein detection

After electrophoresis, proteins were transferred onto an Immobilion^{TM} polyvinylidene fluoride (PVDF) membrane (Millipore, Schwalbach, Germany). The membrane was cut to the desired size (6 x 9.5 cm), rinsed with methanol and equilibrated with transfer buffer (0.193 M Glycin, 0.043 M Tris base, 10% methanol). The gel was placed onto membrane between two pieces of whatman filter paper (7 x 10.5 cm) that was pre-soaked in transfer buffer. No bubbles should be between membrane and gel. The location of gel should be on '-' electrode side, whereas membrane is on '+' electrode side. The sandwich was inserted into the blotting chamber and the blotting chamber was filled with transfer buffer. The transfer was performed at 30 V ON.

The next day, the membrane was incubated with blocking solution (5% milk in PBS/0.02% Tween-20) for 1 hr at RT to reduce unspecific binding of the antibody to the membrane. Thereafter, the membrane was incubated for 1 to 4 hr at RT or ON at 4ºC with a specific primary antibody diluted in blocking solution (see 2.1.4.1) for antibody dilution and incubation time). After incubation with the primary antibody, the membrane was washed three times with PBS/0.02% Tween-20 for 10 min each. Thereafter, the membrane was incubated with a horseradish peroxidise-coupled secondary antibody diluted 1:1000 in blocking solution for 1 hr at RT. The membrane was washed four times with PBST 10 min each. To develop the Western blot, enhancer of chemioluminescence (ECL) solution 1 (2.5 mM of luminol, 0.396 mM of cumaric acid, 0.225 M of Tris, pH 8.5) was mixed 1:1 with ECL solution 2 (0.0192% H2O2, 0.225 M Tris pH 8.5). The membrane was incubated with this solution for 3 minutes. Then the ECL solution was poured off, the membrane was wrapped into cling-film and exposed onto Amersham Hyperfilm ECL (GE-Healthcare, Buckinghamshire, UK) or Fuji Super RX 18x24 film (Ernst Christiansen GmbH, Planegg, Germany).

2.2.3.6 Immunoprecipitation

Cells were washed three times with ice-cold PBS and lysed in NP-40 Lysis buffer, containing 1mM PMSF, 4 mM 1.10-Phenanthroline (Serva) and 10 mM N-ethylmaleimide (NEM) (Sigma). 200 μg of protein were added to ImmunoPure Immobilized Protein A beads pre-coupled with the anti-p53 antibody 421 and the mixtures were incubated 2 hours on a rotating wheel. The precipitates were washed twice with NP-40 Lysis buffer and resuspended in 15 μl of NP-40 Lysis buffer buffer. 15 μl of 2xSDS sample buffer (4% sodium dodecyl sulphate, 0.16 M Tris, pH 6.8, 20 % glycerol, 4% β-mercaptoethanol, 0.002% bromphenol blue) were added to the samples, the samples were heat-denatured and loaded onto a 10% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane in blotting buffer containing 0.1% SDS.

2.2.3.7 Co-immunoprecipation

Cells were washed twice with ice-cold PBS and lysed in Co-IP Lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl), 1 mg of protein was added to ImmunoPure^R Immobilized Protein A beads (Pierce) pre-coupled with the 421 anti-p53 antibody. After 2 hours of incubation on a rotating wheel, the precipitates were washed three times with Co-IP wash buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% Glycerol, 0,1% Triton-X-100) and resuspended in 15 μl of Co-IP wash buffer. 15 μl of 2x SDS sample buffer (4% sodium dodecyl sulphate, 0.16 M Tris, pH 6.8, 20 % glycerol, 4% β-mercaptoethanol, 0.002% bromphenol blue) were added to the samples, the samples were heat-denatured and loaded onto a 10% SDS-PAGE gel.

2.2.3.8 Metabolic labelling

Cells were cultured for 2 hours in DMEM – High Glucose without L-methionine and L-cysteine (Invitrogen), supplemented with 15% dialyzed fetal bovine serum

(PAA), 1% penicillin/streptomycin (Invitrogen). Then cells were pulsed with 150 μCi/ml Cys-Meth mix (Hartmann Analytics) for 15 min. Afterwards, cells were washed with ice-cold PBS, scraped from the dish and lysed in Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 1 mM PMSF). The protein extracts were cleared by centrifugation at 14000 rpm at 4°C for 15 min (Hermle Z233 MK). 100 μl of the 421 (anti-p53) antibody, pre-coupled to ImmunoPure^R Immobilized Protein A beads (Pierce) were added to 500 μg of protein extract, and the mixture was incubated on a rotating wheel at 4°C for 2 hours. The agarose was washed three times with Nonidet P-40 lysis buffer and resuspended in 15 μl of NP-40 buffer. 15 μl of 2x SDS sample buffer (4% sodium dodecyl sulphate, 0.16 M Tris, pH 6.8, 20 % glycerol, 4% β-mercaptoethanol, 0.002% bromphenol blue) were added to the samples, the samples were heat-denatured and loaded onto a 10% SDS-PAGE gel. After blotting the membrane was exposed onto X-ray films.

2.2.3.8 Ubiquitination Assay

R1 cells were transfected with plasmids encoding His-tagged ubiquitin and Mdm2. 24 hours after transfection, cells were harvested. The cells were washed in ice-cold PBS and 10^7 cells were lysed in 6 ml of guanidinium lysis buffer pH 8 (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.01 M Tris, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol). 75μl of Ni^{2+}-nitrilotriacetic acid (NTA)-agarose were added to the lysates, and the mixtures were incubated overnight at room temperature with end-over-end rotation. The NTA-agarose was successively washed with the following buffers: guanidinium buffer (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.01 M Tris, pH 8.0, 10 mM β-mercaptoethanol), urea buffer (pH 8.0, 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.01 M Tris, pH 8.0, 10 mM β-mercaptoethanol), buffer A (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.3, 0.01 M Tris, pH 6.3, 10 mM β-mercaptoethanol), buffer A plus 0.2% triton X-100 and buffer A plus 0.1% triton X-100. Elution was carried out with 60 mM imidazole in 5% SDS, 0.15 M Tris, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol. The eluate was diluted 1:1 with 2x SDS sample buffer and subjected to 10% SDS-PAGE gel. The proteins were transferred to PVDF membrane and probed with the anti-p53 antibody CM5.

3. RESULTS

3.1 p53 level and localization in ES cells and during differentiation

From more recent reports, it is clear that p53 is an important determinant in stem cells (Lin et al. 2005; Meletis et al. 2006; Armesilla-Diaz et al. 2009; Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). However, its regulation in stem cells is less clear. Moreover, while in some papers, p53 was reported to be inactive, others characterized it as a major driver of stem cell differentiation (Lin et al. 2005; Maimets et al. 2008). To make the confusion complete, some authors reported that the normally nuclear transcription factor p53 is localized in the cytoplasm of ES cells and after DNA damage it translocates to the nucleus inefficiently (Aladjem et al. 1998; Hong and Stambrook 2004). Higher level of p53 protein in ES cells was another peculiarity of these cells (Sabapathy et al. 1997; Aladjem et al. 1998; Hong and Stambrook 2004).

After all these uncertainties, I first determined p53 level and intracellular localization in ES cells. I prepared lysates of the ES cell lines D3, CGR8 and R1 as well as of NIH3T3 fibroblasts, which was used as an example of a differentiated cell line and determined p53 abundance by Western blotting. Fig.3.1A clearly shows that p53 is highly abundant in all three ES cell lines while it is hardly detectable in 3T3 cells.

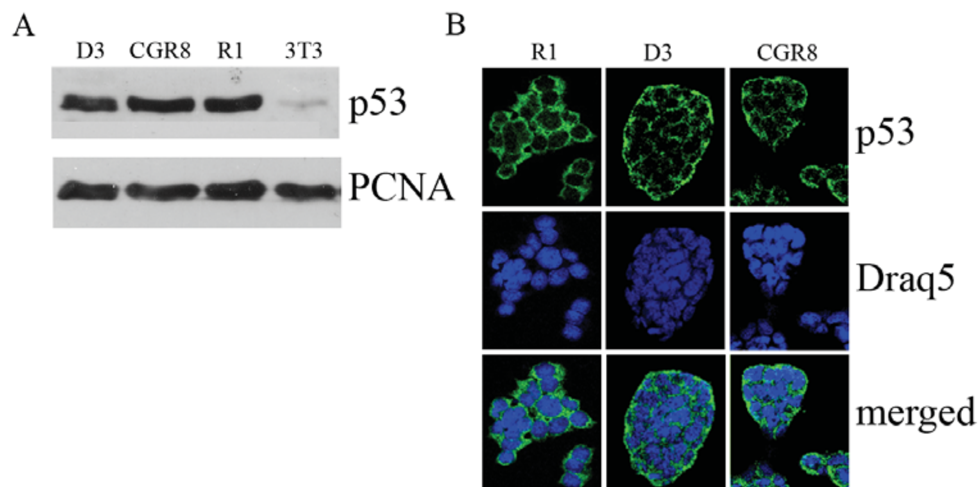

Fig. 3.1 Presence of p53 protein in ES cells and its cytoplasmic localization.

3.1A: D3, CGR8, R1 and NIH3T3 cells were lysed in NP-40 buffer. 50 μg of protein were separated by a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was then incubated with the CM5 (anti – p53 antibody) and PC10 (anti-PCNA) antibody and subsequently with a HRP-coupled anti-mouse antibody. The Western blots were developed with the ECL-method.

3.1B: R1, D3 and CGR8 cells were cultured on gelatine-coated glass slides and fixed with an ice cold acetone-methanol mix. The slides were blocked for 30 min in blocking solution before they were incubated with the. CM5 (anti-p53) antibody. After washing, slides were incubated an anti-mouse antibody coupled with Alexa.488 and with Draq5. After mounting slides were analyzed by Carl Zeiss LSM confocal microscope.

Do determine the localization of p53 in ES cells, R1 and D3 ES cells were grown on gelatin-coated glass slides and stained for p53. Nuclei were visualized by incubation with Draq5. Immunofluorescence microscopy showed that p53 is largely excluded from the nucleus and predominantly present in the cytoplasm of R1 and D3 cells (Fig.3.1B).

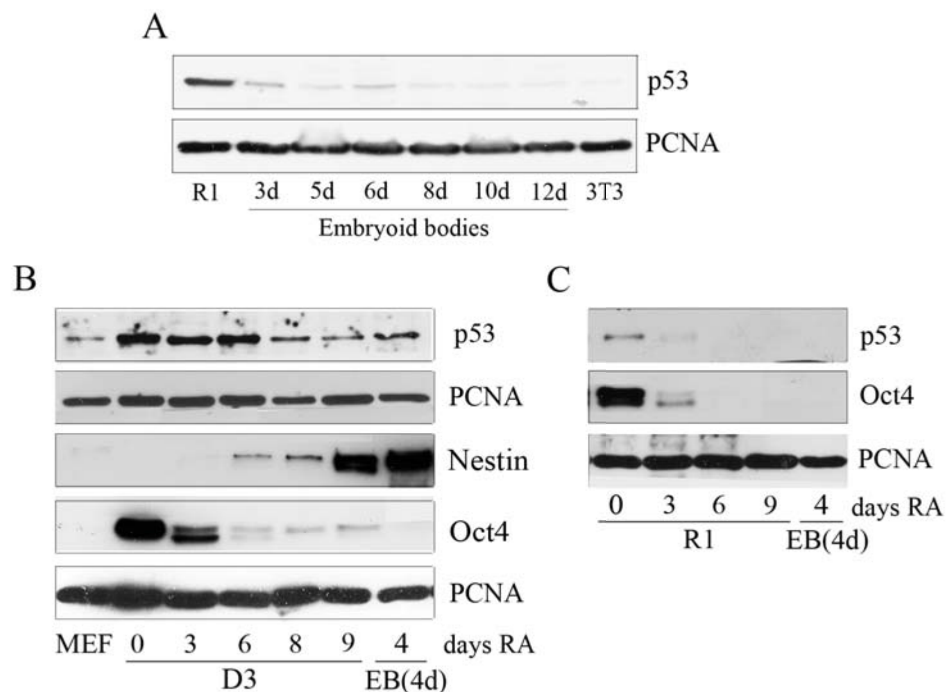

Fig.3.2 P53 is downregulated during differentiation.

3.2A: R1 cells were forced to form embryoid bodies (EB) and harvested at the indicated times. R1 cells, embryoid bodies of the different ages and 3T3 cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The gel was transferred onto a PVDF-membrane and the membrane was probed for the presence of p53 using the Pab 421 (anti-p53) antibody. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method. (d: days).
3.2B: D3 cells or D3-derived embryoid bodies (EB) were incubated with 1 µM all-*trans*-retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The gel was transferred onto a PVDF-membrane and the membrane was probed for the presence of p53 using the Pab 421 (anti-p53). Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. A second membrane was hybridized with the stem cell marker Oct4 and with the differentiation marker nestin. Western blots were developed by the ECL method. (d: days)
3.2C: R1cells and R1-derived embryoid bodies (EB) were incubated with 1 µM retinoic acid (RA) and harvested at the indicated times. R1 and R1-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The gel was transferred onto a PVDF-membrane and

the membrane was probed for the presence of p53 using the Pab 421 (anti-p53) antibody and for anti-Oct4 antibody. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method. (d: days)

Since p53 levels were so much lower in differentiated NIH3T3 cells than in ES cells, p53 must be downregulated at some point during differentiation. To investigate this rationale further, R1 and D3 cells were induced to differentiate by three different protocols, namely by embryoid body (EB) (Maltsev et al. 1994; Rolletschek et al. 2001; Boheler et al. 2002), by treatment with 1µM all-*trans*-retinoic acid (Gu et al. 2005) and by combinations of both (Guo et al. 2001; Bibel et al. 2004; Kim et al. 2009). Embryoid bodies are ES cell-derived three-dimensional multicellular aggregates that resemble early post-implantation embryos (Keller 2005). They contain a wide range of differentiated cell types of all three embryonic germ layers (Dang et al. 2002).

Already at day 3 after differentiation, both by embryoid body formation and by treatment with all-*trans*-retinoic acid, was in R1 cells the abundance of the p53 protein strongly decreased (Fig. 3.2A, Fig. 3.2C). After additional 3 and 6 days of differentiation, p53 protein levels were further reduced and reached approximately the same amount as of mouse embryonic fibroblasts (MEFs) This decline in p53 abundance correlated strongly with a decrease in the stem cell marker Oct4 (Pesce et al. 1998) (Fig. 3.2A, Fig. 3.2C). D3 cells appeared to differentiate slightly slower. Here, Oct4 expression was at 3 days retinoic acid treatment clearly stronger, than in R1 cells with the same treatment and a clear reduction in p53 protein levels was only visible at 8 days after retinoic acid addition. Along with differentiation, the neural precursor marker Nestin (Lendahl et al. 1990) was up-regulated in D3 cells (Fig. 3.2B).

3.2 Degradation of the p53 protein in ES cells

The unusual high level of p53 in ES cells and its anomalous localization raised the question whether degradation of the p53 protein eventually differs between ES cells and differentiated cells.

In differentiated cell lines, p53 abundance is regulated mainly by regulation of its protein stability (Ashcroft and Vousden 1999; Vogelstein et al. 2000). Accordingly, it was very likely that the high abundance of the p53 protein in ES cells would be due to increased protein stability. To investigate this possibility, the half-life of the p53 protein

was determined in ES cells and compared with the half-life of the p53 protein in MEFs. R1, D3 and MEFs were treated with cycloheximide to prevent *de novo* protein synthesis. Cells were harvested after increasing time intervals and the p53 abundance was monitored by Western blotting. After the addition of cycloheximide, the p53 protein disappeared with time and at 1 hour after addition of cycloheximide, it was no longer detectable in both ES cell lines, while it was still present in MEFs (Fig. 3.3A). The intensity of the signals for p53 in R1, D3 and MEF cells were measured, and corrected for eventual loading inconsistencies by using the signals for PCNA. Mean values and standard deviations from three independent experiments were plotted and the half-life of p53 in the different cell lines determined (Fig. 3.3B). The half-life of p53 was about 9 minutes in D3 cells and about 12 minutes in R1 cells. Instead, in MEFs, p53 had a half-life of about 38 minutes (Fig. 6B). Thus, the half-life of the p53 protein in ES cell lines is significantly shorter than the half-life of p53 in differentiated cells.

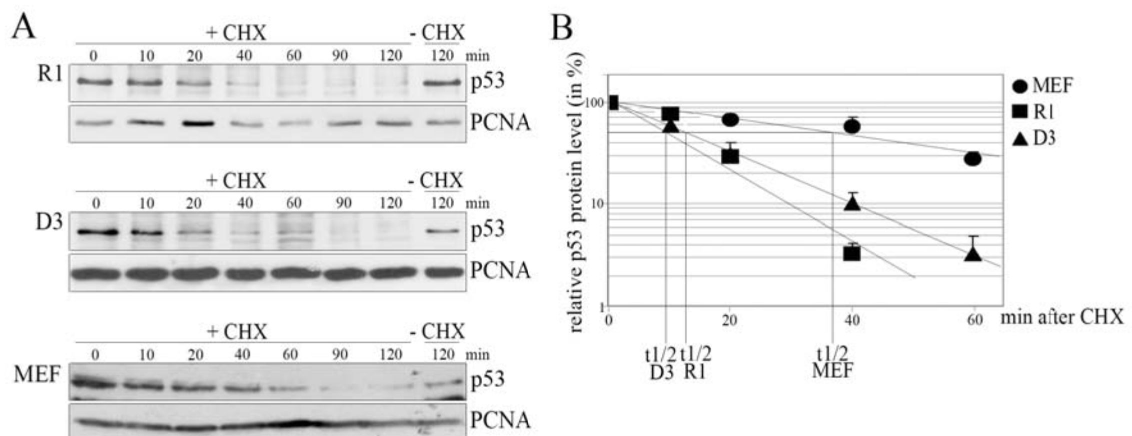

Fig.3.3 Rapid degradation of p53 in ES cells achieved by proteolysis in 26S proteasomes

3.3A: R1, D3 and MEF cells were treated with 50 µg/ml cycloheximide (CHX) and harvested at the indicated times and lysed. 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The gel was transferred onto a PVDF-membrane and the membrane was probed for the presence of p53 using the CM5 (anti-p53) antibody. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.
3.3B: Signals for p53 and PCNA were calculated and mean values and standard deviations of relative p53 expression of three independent experiments were plotted. The half-life of p53 was determined graphically.

In differentiated cells, p53 is degraded through an ubiquitin-dependent pathway in cytoplasmic and nuclear 26S proteasomes (Maki et al. 1996; Freedman et al. 1997; Xirodimas et al. 2001) This degradation pathway requires p53 to travel through the (Yu

et al. 2000). In ES cells, the majority of the protein is localized in the cytoplasm. This unusual localization therefore raised the question whether p53 might also be a target for 26S proteasomes in ES cells. To investigate this issue, R1, D3, and 3T3 cells for control, were treated with the proteasome inhibitor MG132. As shown in figure 3.4, p53, a substrate of 26S proteasomes in differentiated cells, accumulated not only in 3T3 cells in the presence of the proteasome inhibitor, but also in ES cells, indicating that its degradation in ES cells requires functional 26S proteasomes (Fig. 3.4).

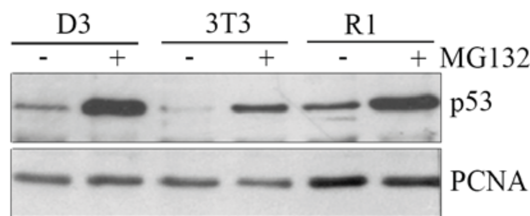

Fig.3.4 Degradation of p53 by 26S proteasome

3.4: R1, D3 and 3T3 cells were treated with the 5µM MG132 or left untreated for control. Cells were harvested after 4 hours and lysed. 50 µg of the proteins were separated by a 10% SDS-PAGE gel and transferred onto a PVDF-membrane. The membrane was probed for the presence of p53 using the Pab 421 antibody. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

In differentiated cells, MDM2 is the major regulator of p53. Mdm2 promotes p53 ubiquitination followed by degradation of the tumour suppressor protein (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997). In differentiated cells, Mdm2 is, like p53, a nuclear protein. Since p53 is in the cytoplasm of ES cells, the question was raised whether Mdm2 might also have such an unusual localisation in ES cells. To investigate this issue, D3 and R1 cells were grown on gelatinised coverslips, fixed and stained for p53 and Mdm2.

In contrast to p53, Mdm2 localises also in ES cells in the cell nucleus (Fig. 3.5A). Thus, p53 and Mdm2 predominantly exist in different subcellular compartments although it cannot be entirely excluded that a minor amount of Mdm2 may also exist in the cytoplasm and that some p53 protein may be present in the nucleus of ES cells.

In differentiated cells, p53 and Mdm2 associate via their N-terminal and central protein domains (Bottger et al. 1997; Midgley and Lane 1997; Chen et al. 1999; Kulikov et al. 2006) and these interactions are required for p53 degradation. The fact that the majority of the p53 and Mdm2 proteins were found in different cellular compartments

raised the question whether (nuclear) Mdm2 is able to regulate (cytoplasmic) p53 in ES cells.

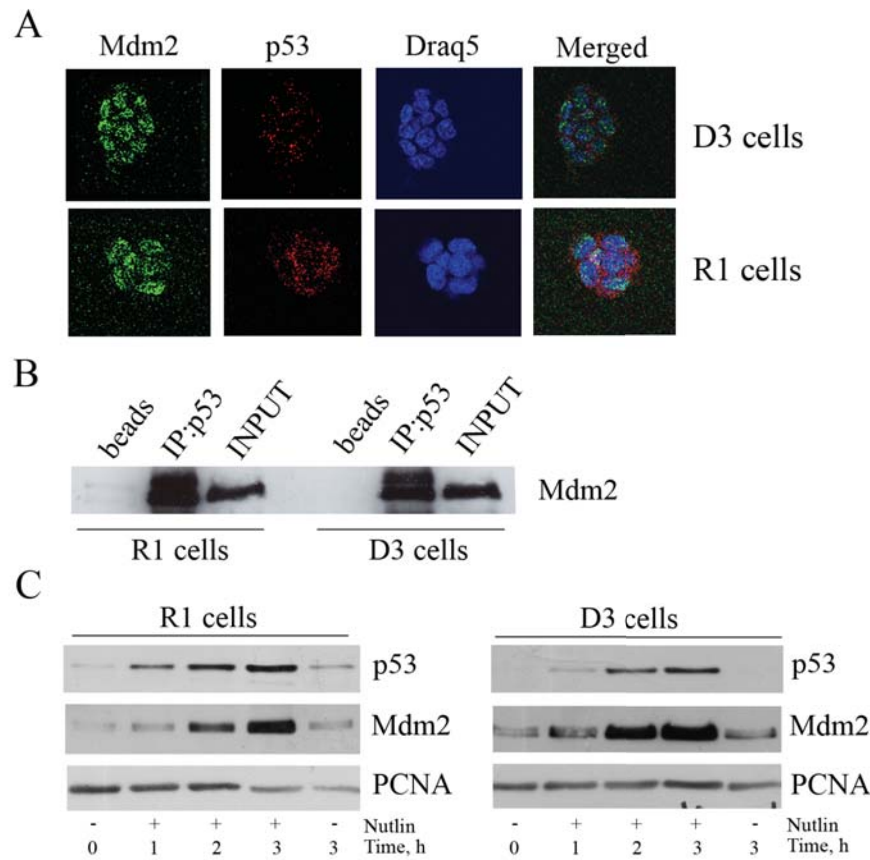

Fig.3.5 Mdm2 regulates p53 degradation in ES cells.

3.5A: R1 and D3 cells were grown on gelatinised coverslips, fixed and incubated with a rabbit anti -p53 antibody (CM5), and with a mouse anti-Mdm2 antibody (4B2). After washing, cells were incubated with an Alexa 488 coupled anti-mouse (green) and with an Alexa-546-coupled anti-rabbit (red) antibody and with Draq5 (blue). Cells were analysed using a Zeiss LSM510 microscope and LSM LSell5 Image Examiner software.
3.5B: R1 and D3 cells were lysed in NP40-buffer and 500 µg of cellular lysate were incubated with Immunopure[R] Immobilized protein A pre-coupled with an anti-p53 antibody. After incubation for 2 hours, the protein A/antibody/p53 precipitates were pelleted, washed and loaded onto a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane and the membrane was incubated with an anti-p53 antibody. The Western Blot was developed by the ECL method.
3.5C: R1 and D3 cells were treated with 10µM nutlin and harvested at the indicated times. 50 µg of the proteins were separated by a 10% SDS-PAGE gel and transferred onto a PVDF-membrane. The membrane was probed for the presence of p53 using the Pab 421 antibody and for Mdm2 using the 4B2 antibody. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

To address this question, co-immunoprecipitation experiments were performed. R1 and D3 cells were lysed and p53 was precipitated using an anti-p53 antibody. Then

associated Mdm2 was determined by Western blotting. As shown in Figure 3.5B, p53 and Mdm2 from R1 and D3 cells associated with each other. However, it must be noted, that detection of Mdm2 in p53-immunoprecipitates could also be due to secondary interaction of Mdm2 and p53 after lysis. Therefore, the interaction of p53 and Mdm2 was further investigated by functional approaches. One possibility to test the interaction of p53 and Mdm2 is by treating cells with nutlin. Nutlin is a compound that binds to Mdm2 and prevents the interaction of p53 and Mdm2 (Vassilev et al. 2004). Consequently, p53 accumulates in the presence of Nutlin. When R1 and D3 cells were treated with Nutlin, p53 accumulated to high levels (figure 3.5C). It should be noted that an increase of Mdm2 was also observed under these conditions (figure 3.5C), indicating that p53 was transcriptionally active.

Since Mdm2 is an E3 ubiquitin ligase for p53, this function of Mdm2 was also tested in ES cells. Figure 3.6 shows results of ubiquitination assay that was performed in R1 cells. Co-transfection of Mdm2 together with the Ubiquitin-encoding construct led to increase of p53 ubiquitination (Fig.3.6).

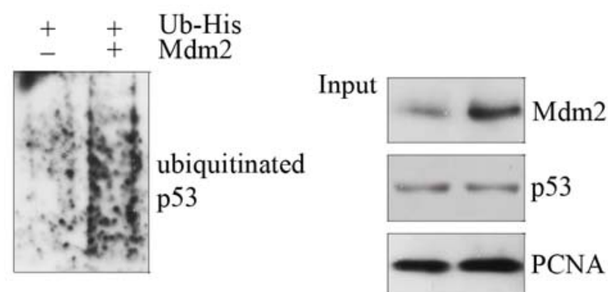

Fig.3.6 Mdm2 ubiquitinates p53 in ES cells

3.6 R1 cells were transfected with plasmids encoding His-tagged ubiquitin and Mdm2, or with plasmids encoding His-tagged ubiquitin and vector DNA for control. 24 hours after transfection, cells were harvested. An aliquot of the cells (1/10) was lysed in NP40 buffer and levels of Mdm2, p53 and PCNA were analysed by Western Blotting. (Input). The rest of the cells was lysed in guanidinium buffer and incubated overnight with Ni-NTA-agarose. Ubiquitinated proteins were eluted and p53 levels determined by Western blotting using the anti-p53 antibody Pab421 (ubiquitinated p53).

Although Mdm2 is the major regulator of p53, at least in differentiated cells (reviewed in (Boehme and Blattner 2009)) it is not the only ubiquitin ligase for p53. Since the ubiquitin ligase PirH2 has also been shown to target p53 for proteasomal degradation (Leng et al. 2003), it was tested whether Pirh2 may also regulate p53 in ES cells. First, it was investigated whether PirH2 levels are changed during differentiation.

Therefore, D3 cells were differentiated by incubation with 1µM retinoic acid or by formation of embryonic bodies, combined with treatment with 1µM retinoic acid and expression of PirH2 was compared between MEFs, D3 cells and differentiated D3 cells. As shown on the Western Blots of Fig. 3.7A, there was no change in the level of PirH2 during differentiation. To determine the influence of PirH2 on p53 abundance in ES cells, PirH2 level were decreased in R1 cells by transfecting a siRNA targeted against PirH2 RNA. Thereafter p53 abundance was determined by Western blotting. However, despite a significant reduction of Pirh2 protein levels after transfection of a PirH2-siRNA, the p53 protein did not accumulate (Fig. 3.7B).

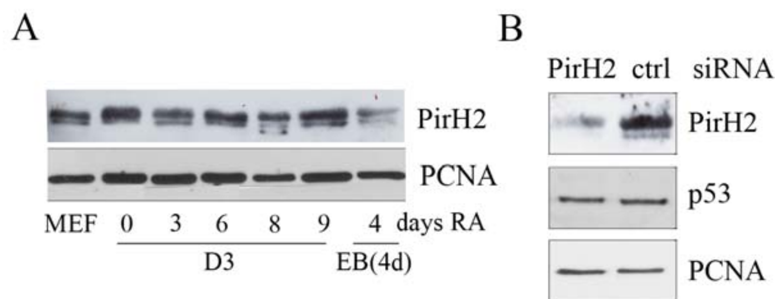

Fig.3.7 P53 is not regulated by PirH2 in ES cells

3.7A: D3 cells or D3-derived embryoid bodies were incubated with 1µM all-*trans*-retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of PirH2. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control.
3.7B: D3 cells were transfected with a siRNA directed against Pirh2 RNA or with a control siRNA. 48 hours after transfection, cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of PirH2 and p53. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

Ubiquitination of a target protein can also be reverted. This reaction is catalysed by specific ubiquitin hydrolases. The p53 protein can be deubiquitinated by the ubiquitin protease Hausp and overexpression of Hausp has been shown to stabilize p53 in a lung carcinoma cell line and in mouse embryos (Li et al. 2002).

To determine the level of Hausp during differentiation of ES cells, D3 cells were forced to differentiate by incubation with retinoic acid and by a combination of embryoid body formation and incubation with retinoic acid. Samples were taken at different time point during differentiation and abundance of Hausp was determined by Western Blotting.

As shown in Figure 3.8A, the level of Hausp was not altered during differentiation (Fig. 3.8A). To investigate whether Hausp has a functional impact on p53 abundance in ES cells, Hausp was overexpressed as well as downregulated by siRNA.

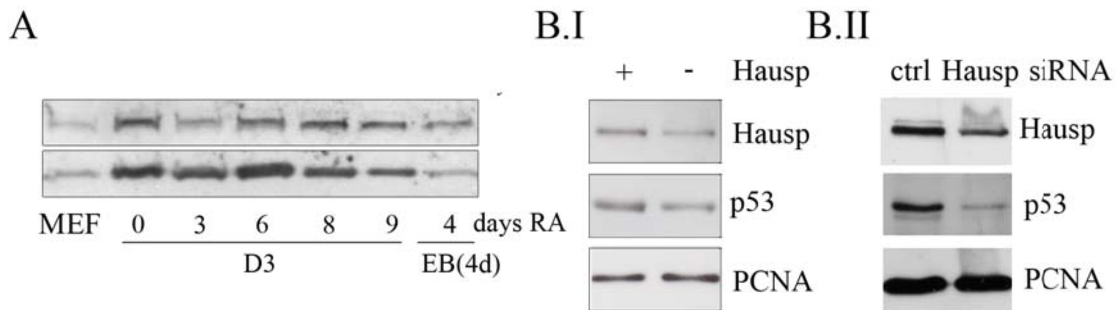

Fig.3.8 P53 is regulated by Hausp in ES cells

3.8A: D3 cells or D3-derived embryoid bodies were incubated with 1µM retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of Hausp. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control.
3.8B: D3 cells were transfected with a plasmid expressing Hausp or with a vector control **(I)** or with a siRNA directed against Hausp RNA or with a control siRNA **(II)**. 24 hours after plasmid transfection or 48 hours after siRNA transfection, cells were lysed and 50 µg of the proteins were separated by 10% SDS-PAGE gels. The proteins were transferred onto PVDF-membranes and the membranes were probed for the presence of Hausp and p53. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

While overexpression of Hausp led to an increase in p53 abundance, p53 levels were decreased when the amount of Hausp protein was decreased (Fig. 3.8B), demonstrating that Hausp controls p53 abundance also in ES cells

Ubc13 is an ubiquitin-conjugating enzyme that attenuates Mdm2-dependent degradation of p53 and regulates translocation of p53 to the cytoplasm (Laine et al. 2006). Because of this important role of Ubc13, Ubc13 protein levels were monitored during differentiation. D3 cells were differentiated by incubation with retinoic acid and by a combination of embryoid body formation and retinoic acid. Samples were taken at different time point during differentiation and abundance of Ubc13 was determined by Western Blotting.

As shown in Figure 3.9A, Ubc13 abundance was not altered between non-differentiated and differentiated ES cells or MEFs, indicating that levels of Ubc13 do not correlate with the differentiation state of cells (Fig.3.9A).

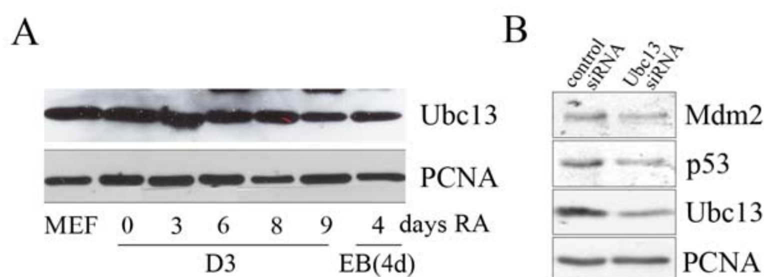

Fig.3.9 P53 is regulated by Ubc13 in ES cells

3.9A: D3 cells or D3-derived embryoid bodies were incubated with 1µM retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of Ubc13. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control.
3.9B: D3 cells were transfected with a siRNA directed against Ubc13 RNA or with a control siRNA. 48 hours after transfection, cells were lysed and 50 µg of the proteins were separated by 10% SDS-PAGE gels. The proteins were transferred onto PVDF-membranes and the membranes were probed for the presence of Ubc13 and p53. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

To test whether Ubc13 is functionally implicated in the regulation of p53 in ES cells, Ubc13 was downregulated in R1 cells by siRNA. Transfection of a siRNA targeted against Ubc13 clearly reduced Ubc13 protein levels. This decrease in Ubc13 went along with a clear decrease in p53 abundance, showing that Ubc13 is an integral component of the regulation of p53 protein abundance in ES cells (Fig. 3.9B).

3.3 Regulation of p53 in ES cells occurs at the level of RNA stability and translation

Since the stability of the p53 protein was significantly lower in ES cells than in differentiated cells (Fig. 3.3A) increased protein stability cannot account for the high abundance of the p53 protein in ES cells. It was therefore highly possible that ES cells might have higher levels of p53 RNA or that p53 RNA may be translated more efficiently. To investigate these issues, RNA was prepared from several samples and different passages of R1, D3, MEF cells and differentiated R1 and D3 cells. Then RNA was transcribed into cDNA and qRT-PCR was performed.

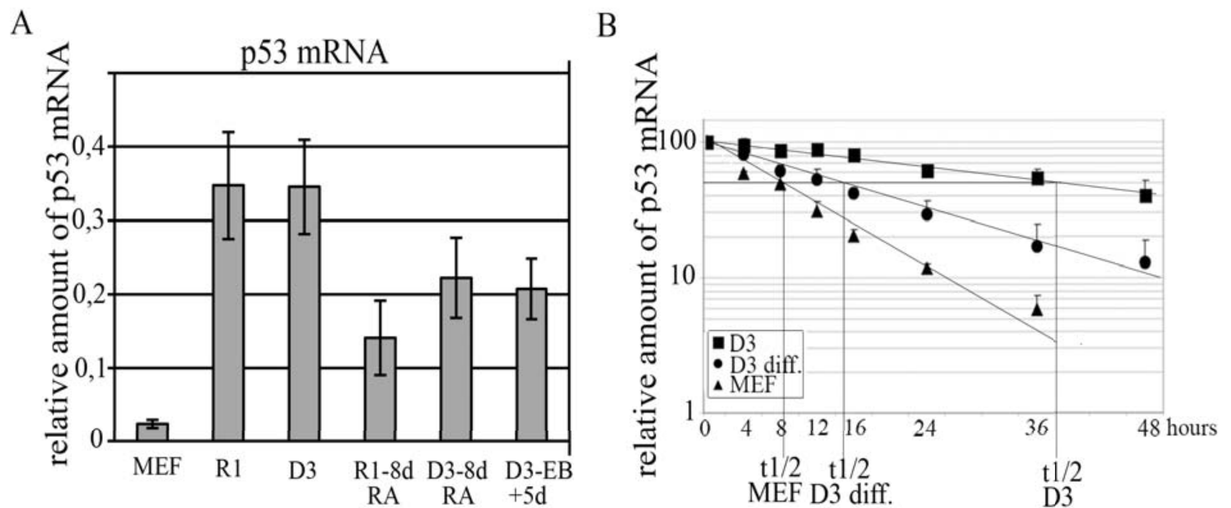

Fig.3.10: P53 mRNA abundance and stability in ES cells and in differentiated cells

3.10A: Total RNA was prepared from MEF, R1, D3, and from R1 and D3 cells differentiated for eight days with 1 µm retinoic acid (RA) or from D3 cells differentiated by embryoid body formation and treatment with 1 µM retinoic acid (RA) and used for cDNA synthesis. The resulting cDNAs were analysed for p53 abundance by qRT-PCR using p53-specific primers as well as primers for the housekeeping gene RibPO (34b4). ΔCT values were calculated and the values for p53 were normalised by the values for RibPO. Mean values for the relative amount of p53 and standard deviations of three independent experiments were plotted.

3.10B: MEF, D3, D3 that had been differentiated by incubation with 1µM of retinoic acid for eight days (d3-8d RA) were treated with 50 µg/ml of Actinomycin D and harvested at the indicated time points. RNA was extracted from these samples and cDNAs were synthesized. P53 mRNA level was analysed by qRT-PCR and signals were normalized to RibPO expression level. Mean values and standard deviation of three independent experiments were plotted. The relative amount of p53 RNA at the time when actinomycin D was added was set to 100.

As shown in figure 3.10A, R1 and D3 cells contained about fourteen times more p53 RNA than MEFs (Fig. 3.10A). When these ES cells are induced to differentiate, the amount of p53 becomes reduced by one third (D3) or even by two thirds (Fig. 3.10A).

Such an increase in the amount of a specific mRNA can be due to increased stability of the mRNA or to increased gene transcription. To investigate which of these principles is responsible for the higher amount of p53 RNA in ES cells, the stability of p53 RNA was determined. D3, D3 cells differentiated for eight days by incubation with retinoic acid and MEF cells were incubated with actinomycin D to block transcription. The decay of p53 RNA was then monitored by qRT-PCR.

For all cells, a continuous decrease of p53 RNA was observed. However p53 mRNA from MEF and differentiated D3 cells decayed significantly faster. For MEFs, a

half-life of 8 hours was calculated for p53 RNA. The half-life of p53 RNA in differentiated D3 cells was about 15-16 hours while in non-differentiated D3 cells it was more than 36 hours (Fig. 3.10B).

Taken together, these results show that stem cells contain more p53 mRNA than differentiated cell. Moreover, p53 mRNA was more stable in stem cells. Unfortunately, it was not possible to compare the rate of p53 gene transcription between stem cells and differentiated cells. Probably the rate of p53 transcription is too low to be determined by conventional assays.

Higher amount of p53 mRNA may lead to higher translation rates of p53 protein and result in higher protein level. To investigate this issue in more detail, MEF, R1, D3, and R1 and D3 cells that been differentiated by incubation with retinoic acid for eight days were incubated with ^{35}S-methionine/cysteine for 15 min. p53 was immunoprecipitated and the relative amount of incorporated radioactivity was determined according (Bonifacino 2001).

Within the 15-minute labelling time, R1 and D3 cells synthesized significantly more p53 protein than differentiated cells (Fig. 3.11A, B). The signals on the autoradiogram strongly suggest a higher rate of p53 translation for R1 and D3 stem cells than for MEF or differentiated R1 and D3 stem.

To further investigate the reason for the increased stability of p53 RNA in ES cells, the level of microRNAs was determined. MicroRNAs (miRNAs) are a class of non-coding RNAs that emerged as important regulators of gene expression (Bartel 2004). Recently two isomers of miRNA125, miRNA-125a and miRNA125b, have been shown to reduce p53 expression (Le, Teh et al. 2009; Zhang, Gao et al. 2009). MiRNA-125b binds p53 mRNA on a microRNA-response element in the 3’ UTR and reduces p53 protein levels. This decrease in p53 expression is critical for controlling the rate of apoptosis during development as well as during the stress response in human cells and zebrafish embryos (Le et al. 2009). Like miRNA125b, miRNA-125a binds to the 3’UTR of p53 RNA and represses p53 translation (Zhang et al. 2009).

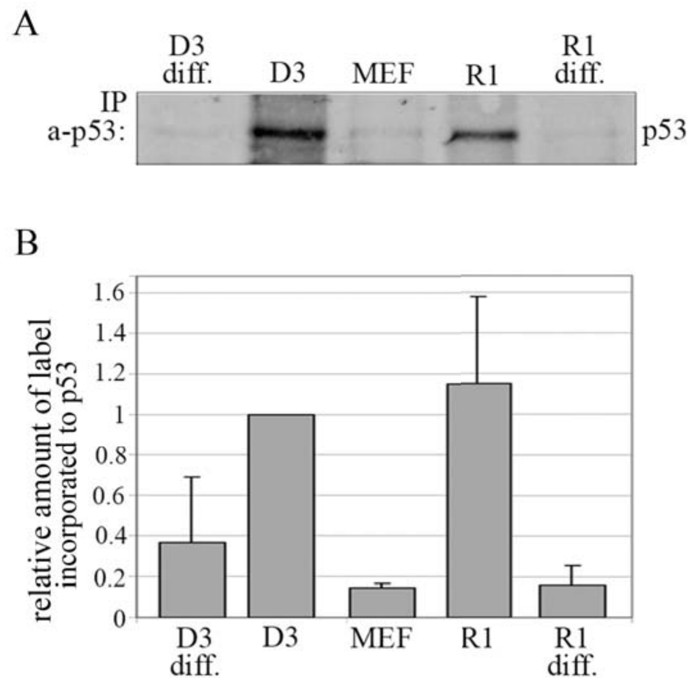

Fig.3.11: More p53 is translated in stem cells

3.11A: Cells were grown for two hours in DMEM medium without L-cysteine and L-methione, supplemented with dialysed foetal calf serum. Afterwards, cells were pulse-labelled with 150 µCi/ml of ^{35}S-Cys/methionine for 15 min. Cell lysates were prepared and p53 was precipitated using the anti-p53 antibody Pab421 pre-coupled to ImmunoPure^{R} Immobilized Protein A. The precipitates were washed and loaded onto a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane and the membrane was exposed onto an X-ray film.

3.11B: The signals of the X-ray film were measured; mean values and standard deviation of three independent experiments were calculated and plotted. The value for undifferentiated D3 cells was set to 1.

To compare expression of miRNA-125b and miRNA125b of stem cells with that of differentiated cells, RNA was prepared from the ES cell lines R1, D3 and CGR8, from MEF and from R1 and D3 cells as well as from D3-derived embryoid bodies that had been incubated with retinoic acid to foster differentiation. cDNAs of micro-RNA 125a and micro-RNA 125b were synthesized by using primers specific for miRNA-125a or miRNA-125b.

Figure 3.12A shows that both microRNAs (miRNA-125a and miRNA-125b) are expressed at much lower amounts in the three ES cell lines (R1, D3, CGR8) than in differentiated R1 or D3 cells or in MEFs (Fig.3.12A).

To further investigate whether the lower amount of micro-RNAs in ES cells is just co-incidence, or whether there is indeed a functional relation, micro RNAs miRNA-125a and miRNA-125b were cloned and R1 cells were transfected with plasmids encoding micRNA-125a, microRNA-125b, or null vector. As the Western Blot shown in

Figure 13B shows, overexpression of either microNA-125b or microRNA-125a resulted in a decrease of p53 protein levels in R1 cells. The right panel of this figure shows specific expression of miRNA-125a or miRNA-125b after overexpression (Fig.3.12B).

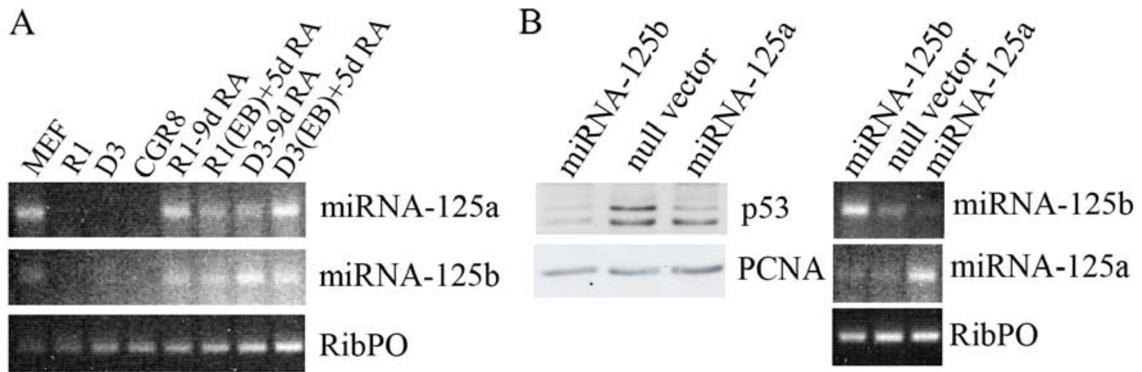

Fig.3.12 MicroRNA miRNA-125a and miRNA-125b levels in ES cells and in differentiated cells.

3.12A: RNA was prepared from MEFs, from the ES cell lines R1, D3 and CGR8, from R1 and D3 cells that had been incubated for 8 days with 1µM all-*trans*-retinoic acid (R1-9d RA; D3-9d RA) and from R1 and D3-derived embryoid bodies that have been further differentiated by incubation with 1µM all-*trans*-retinoic acid for 5 days (R1(EB)+5d RA; D3(EB)+5d RA) cDNAs were synthesized using miRNA-125a and miRNA-125b specific primers to synthesize miRNA-125a cDNA and miRNA-125b cDNA and by using random primers for general cDNA synthesis. miRNA-125a and miRNA-125b were further amplified by PCR using gene specific primers. RibPO that was used for control was amplified from the total cDNA pool by using primers specific for RibPO. All PCR products were analyzed by agarose gel electrophoresis.
3.12B: R1 cells were transfected with plasmids encoding miRNA-125a, miRNA-125b or with vector for control. 24 hours after transfection half of the cells were lysed and analyzed by Western blotting for p53 expression as described in the legend to figure 9b (left panel). The remaining cells were lysed and RNA was prepared. The RNA was analyzed for expression of miRNA-125a and for miRNA-125b as described in the legend to part A of this figure.

This decrease in p53 abundance is at least in part due to a decrease in p53 RNA level and stability (Fig 3.13A). Fig.3.12A shows the levels of p53 mRNA after actinomycin D treatment in R1 cells overexpressing miRNA-125a and miRNA-125b or R1 cells transfected with vector control. Overexpression of both miRNA-125 resulted in less p53 mRNA stability. Consistently, overexpression of miRNA-125a and miRNA-125b reduced p53 RNA levels by about 20% over a twenty-four-hour time period (Fig.3.13B). Concomitantly with the decrease in p53 RNA, a remarkable decrease in the incorporation of radioactivity into p53 protein was observed after a fifteen-minute pulse with ^{35}S-cysteine/methionine (Fig 3.13D). Transfection of miRNA-125a and miRNA-125b reduced *de novo* p53 protein synthesis by about 60% (Fig. 3.13D). Since the

amount of p53 RNA was only reduced by 20% within this time frame (Fig.3.13B), it is most likely that these mircoRNAs also affect the rate of p53 translation (Fig. 3.13E).

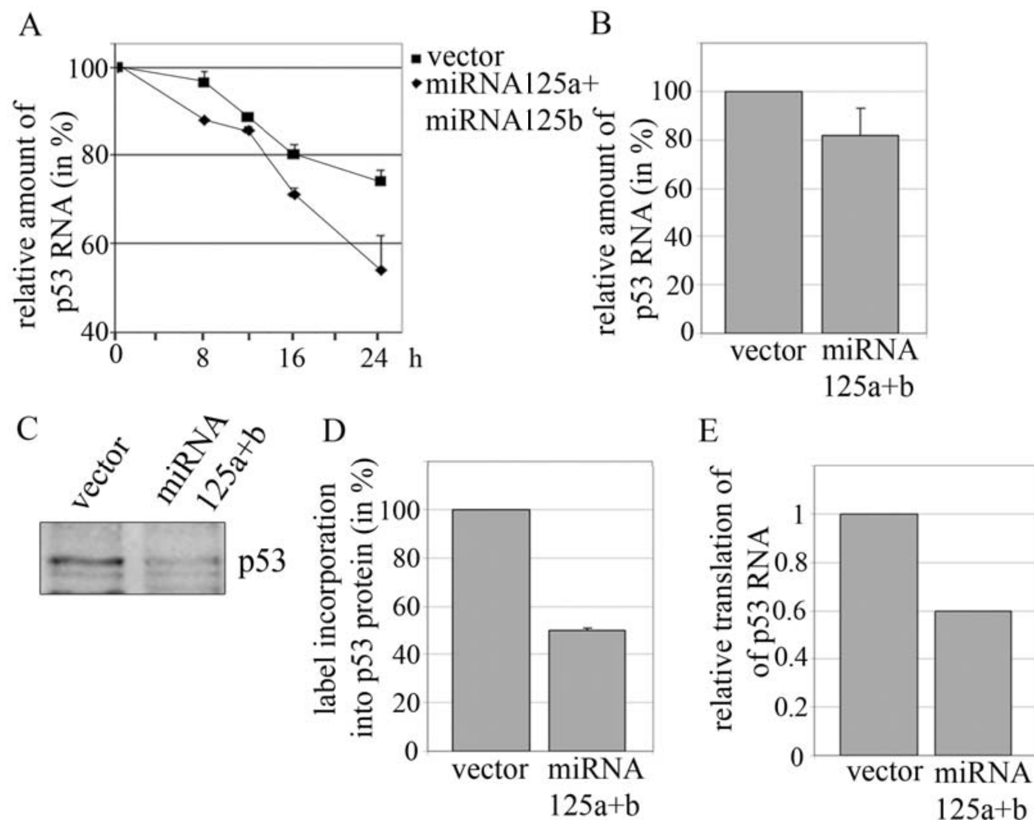

Fig. 3.13 Regulation of p53 translation by miRNA-125a and b

3.13A: R1 cells were transfected with plasmids expressing miRNA-125a and miRNA-125b or with vector for control. 24 hours after transfection, cells were treated with 5 µg/ml actinomycin D, or with DMSO for control and harvested at the indicated time points. RNA was extracted and qRT-PCR was performed for p53 and the housekeeping gene RibPO (34B4). The signals that were obtained for p53 were corrected by the signals for the RibPO and mean values for the relative amount of p53 RNA and experimental variation were calculated from 2 independent experiments and plotted. The value for the relative amount of p53 RNA at the time of actinomycin D addition (0) was set to 100%.

3.13B: R1 cells were transfected with plasmids expressing microRNA miRNA-125a and miRNA-125b or with vector for control. 24 hours after transfection, cells were harvested, RNA was extracted and qRT-PCR was performed for p53 and the housekeeping gene RibPO (34B4). The ΔCT signals that were obtained for p53 were corrected by the signals for the RibPO and mean values for the relative amount of p53 RNA and experimental variation were calculated from 2 independent experiments and plotted. The value for the relative amount of p53 RNA from vector-transfected cells was set to 100%.

3.13C: R1 cells were transfected with plasmids expressing miRNA-125a and miRNA-125b or with vector for control. 24 hours after transfection, cells were pulse-labelled for 15 minutes with 150 µCi/ml of a ^{35}S-methionine-cysteine mix. P53 was immunoprecipitated with the Pab 421 antibody pre-coupled to Immunopure^{R} Immobilized protein A and loaded onto a 10% SDS-PAGE gel. The proteins were blotted onto a PVDF membrane and exposed against an X-ray film

3.13D: The signals of the X-ray film were measured; mean values and experimental variation of two independent experiments were calculated and plotted. The value for vector-transfected cells was set to 1.

3.13E: The ratio of the relative amount of p53 RNA (B) and the relative label incorporation (D) was calculated and blotted. The value of the ration for vector-transfected cells was set to 1.

Translation of p53 is furthermore controlled by the ribosomal protein L26 (Takagi et al. 2005). L26 binds preferentially to the 5'UTR of p53 RNA and enhances the association of p53 RNA with polysomes resulting in increased p53 translation. Accordingly, overexpression of L26 increased the amount of p53 in several human cell lines as well as in the murine cell line Baf-3 (Takagi et al. 2005).

Since ES cells possess a higher amount of p53 protein and a higher rate of translation (Fig. 3.1A and Fig. 3.11), L26 levels were studied in ES cells and in differentiated cells.

Therefore, samples were taken from MEFs, D3 cell, from D3 cells that were differentiated by incubation with retinoic acid and from D3-derived embryoid bodies that were further differentiated by incubation with retinoic acid for additional five days. These samples were analysed for L26 abundance by Western blotting.

As shown in figure 3.14A, L26 protein level did not differ among the different cell lines and differentiation states (Fig. 3.14A). This result, though, raised the question, whether L26 would at all be able to regulate p53 abundance in ES cells.

To address this question, L26 was overexpressed in R1 and NIH3T3 cells and p53 abundance was determined by Western blotting. In addition, L26 was downregulated in R1 cells. R1 cells with downregulated L26 protein were also assayed for p53 expression.

Interestingly, when R1 and 3T3 cells were transfected with a cDNA encoding L26 and p53 protein levels were monitored by Western blotting, p53 protein levels were strongly elevated in R1 cells that had been transfected with L26, while such an increase in p53 abundance in 3T3 cells was not observed despite similar transfection efficiencies (Fig. 3.14B)

Consistently, when L26 was downregulated in R1 cells, it resulted in decreased p53 levels (Fig.3.14C).

Taken together, miRNA-125a and miRNA125b overexpression or L26 knockdown led to decreased p53 protein levels, indicating that translational control of p53 is necessary to maintain higher amounts of p53 protein in ES cells

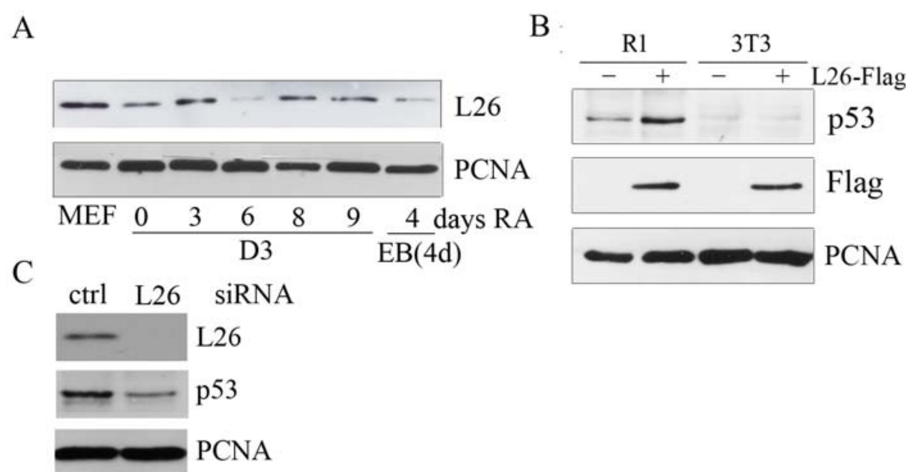

Fig.3.14: L26 protein is functional in ES cells

3.14A: D3 cells or D3-derived embryoid bodies were incubated with 1µM all-*trans*-retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of L26. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.
3.14B: R1 and 3T3 cells were transfected with a plasmid encoding L26-Flag. 24 hours after transfection, cells were lysed and 50 µg of the proteins were separated by 10% SDS-PAGE gels. The proteins were transferred onto PVDF-membranes and the membranes were probed for the presence of L26 (anti-Flag) and p53. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.
3.14C: D3 cells were transfected with a siRNA directed against L26 RNA or with a control siRNA. 48 hours after transfection, cells were lysed and 50 µg of the proteins were separated by 10% SDS-PAGE gels. The proteins were transferred onto PVDF-membranes and the membranes were probed for the presence of L26 and p53. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method. .

3.4 Regulation of cytoplasmic localization of p53 in ES cells

In addition to the arbitrarily high level of p53 in ES cells, the p53 protein is also localised in the cytoplasm. The molecular basis for this unusual localisation of the p53 protein in ES cells is as yet unclear.

Similar to ES cells, p53 is also sequestered in the cytoplasm of several cancer cell lines (Sun et al. 1992; Ueda et al. 1995; Moll et al. 1996; Lou et al. 1997; Schlamp et al. 1997) and a number of explanations have been proposed to account for this cytoplasmic localization. Examples are overexpression of Mortalin, a Hsp70 family member, or of Parc, a parkin-like ubiquitin ligase (Wadhwa et al. 2002; Wadhwa et al. 2002; Nikolaev et al. 2003; Dundas et al. 2005).

Obviously, the localisation of p53 must change during differentiation since otherwise it could not be explained why p53 is cytoplasmic in ES cells and nuclear in differentiated cells. Along this rationale, Parc and Mortalin protein levels were tested in ES cells and in differentiated cells.

Therefore, samples were taken from MEFs, D3 cell, from D3 cells that were differentiated by incubation with retinoic acid and from D3-derived embryoid bodies that were further differentiated by incubation with retinoic acid for additional five days. These samples were analysed for the abundance of Parc and Mortalin by Western blotting.

As it is shown in figure 3.15, there was no difference in Mortalin expression between D3 ES cells, D3-derived differentiated cells or MEFs (Fig. 3.15). In contrast to this very similar expression of Mortalin in all investigated cell lines and derivatives, Parc expression was significantly lower in primary mouse fibroblasts than in D3 ES cells. However, since the expression of Parc is not decreased when D3 cells were forced to undergo differentiation (Fig. 3.15), it is more likely that the low expression of Parc in MEFs is specific for this particular cell type and not for differentiated cells in general.
Since Parc and Mortalin have been shown to interact with p53 (Wadhwa et al. 2002; Nikolaev et al. 2003), it was investigated whether there is a difference in the quantity of the formed complexes between ES and differentiated cells. However no interaction between Parc and p53 or with Mortalin and p53 could be detected (data not shown).

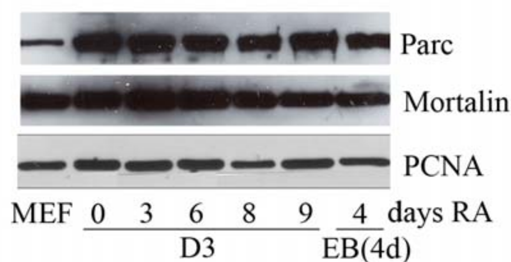

Fig.3.15 Mortalin and Parc expression in ES cells and in differentiated cells

3.15: D3 cells or D3-derived embryoid bodies were incubated with 1µM all-*trans*-retinoic acid (RA) and harvested at the indicated times. MEF, D3 and D3-derived differentiated cells were lysed and 50 µg of the proteins were separated by a 10% SDS-PAGE gel. The proteins were transferred onto a PVDF-membrane and the membrane was probed for the presence of Parc and Mortalin. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

Apart from its abundance, functionality of a protein can also be regulated by its subcellular localization. Accordingly, localisation of Parc and Mortalin was assayed in

ES cells and in differentiated cells. R1 cells were grown on gelatinised cover slips, stained for Parc and Mortalin and analysed by immunofluorescence microscopy (Fig.3.16).

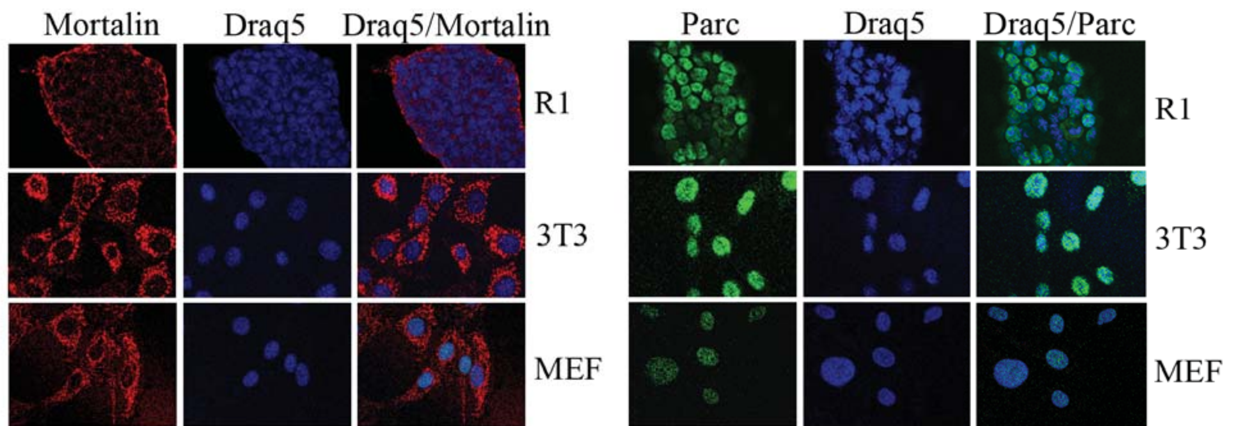

Fig.3.16 Localization of Mortalin and Parc in different cell lines

3.16: R1 cells, grown on gelatinized coverslips and NIH3T3 cells and MEFs, grown on coverslips, were fixed in acetone/methanol and incubated overnight with antibodies targeted against Mortalin and Parc, or with vehicle for control. After washing, coverslips were incubated with an antibody directed against rabbit IgG coupled to Alexa-Fluor-546 (red) or with an antibody directed against mouse IgG coupled to Alexa-Fluor-488 (green) and with Draq5 (blue) to visualize the nuclei. After mounting slides were analyzed by Carl Zeiss LSM confocal microscope.

Consistent with previous observations in differentiated cells (Kaul, Yaguchi et al. 2003; Wadhwa, Ando et al. 2003) Mortalin was localised in the cytoplasm of R1, NIH3T3 and MEF cells while Parc showed a nuclear localisation in all three cell lines.

Thus there is no obvious difference in the sub-cellular localization of Mortalin and Parc between ES and differentiated cells.

Since it was reported from p53 that it can be ubiquitinated by Ubc13, which is a ubiquitinating enzyme that predominantly links ubiquitin chains via lysine 63 (Laine et al. 2006) and that this unusual linkage of ubiquitin molecules on the p53 protein keeps p53 in the cytoplasm, it was of particular interest to see whether ubiquitination of p53 in ES cells differs from ubiquitination of p53 in differentiated cells.

D3 cells and differentiated D3-8d RA cells were used for immunoprecipitation. Moreover, D3 cells were treated by IR to see any changes in p53 ubiquitination state between irradiated and non-irradiated cells, because it had been shown that p53 translocates to the nucleus after irradiation, and it might be reflected on its

ubiquitination state. Western blot analysis of immunoprecipates does not show any differences between differentiated D3 and D3. However, irradiation led to decrease of K63-ubiquitination both in the presence and the absence of proteasome inhibitor MG132, while p53 level was increased (Fig. 3.17). Although, it was not checked where p53 located in D3-8d RA differentiated cells, it was shown that after 2 hours post-irradiation by 7.5 Gy dose, p53 locates into the nucleus of ES cells (Fig.3.18). Therefore, this K63-ubiquitination might regulate p53 cytoplasmic localization.

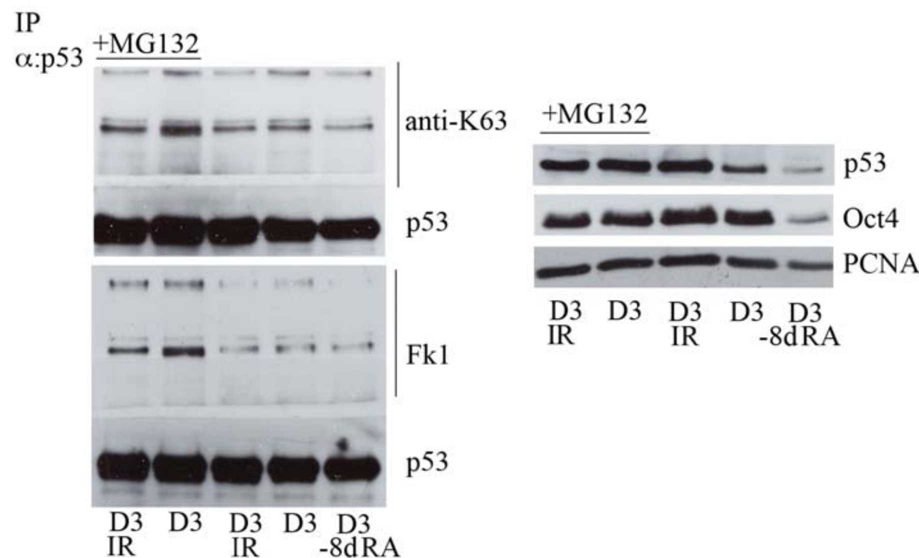

Fig.3.17 Ubiquitination state of p53 in ES cells

3.17: D3 cells, D3-8d RA differentiated cells, D3 cells treated with 7,5 Gy of ionizing radiation (IR), treated by MG132, or together by IR and MG132 were harvested after 2 hours post-irradiation, or 4 hours after MG132 treatment. Cellular extracts were precipitated to anti-p53 (Pab 421) antibody pre-coupled to Immunopure^R Immobilized protein A. Precipitates were loaded onto SDS-PAGE and western blot was performed using antibodies recognizing K63-linked ubiquitin chains (k63) and Fk1, which recognizes poly- and mono-ubiquitinated proteins (left panel). Right panel: input control for immunoprecipitation (IP), 50 μg of protein extracts were analyzed by western blotting using anti-p53 (421), anti-Oct4 and anti-PCNA antibodies.

3.5 p53 activity after DNA damage in ES cells

In response to DNA damage p53 accumulates in the nucleus of differentiated cells and activates transcription of its target genes. Because of its important role, that p53 has in the DNA damage response of differentiated cells it is likely that p53 has a

similar function in ES cells. However, the data whether p53 is active or not in ES cells after DNA damage are contradictory and confusing.

Since in some publications, p53 was reported to be in the cytoplasm of ES cells, it was of particular interest to investigate whether it accumulates at all in response to DNA damage and if yes, whether it accumulates in the cytoplasm or in the nucleus of ES cells.

To investigate the subcellular localization of p53 during the DNA damage response, R1 cells were grown on gelatinized cover slips, irradiated with 7.5 Gray of γ-irradiation and stained for p53. The data were then analyzed by immunofluorescence microscopy.

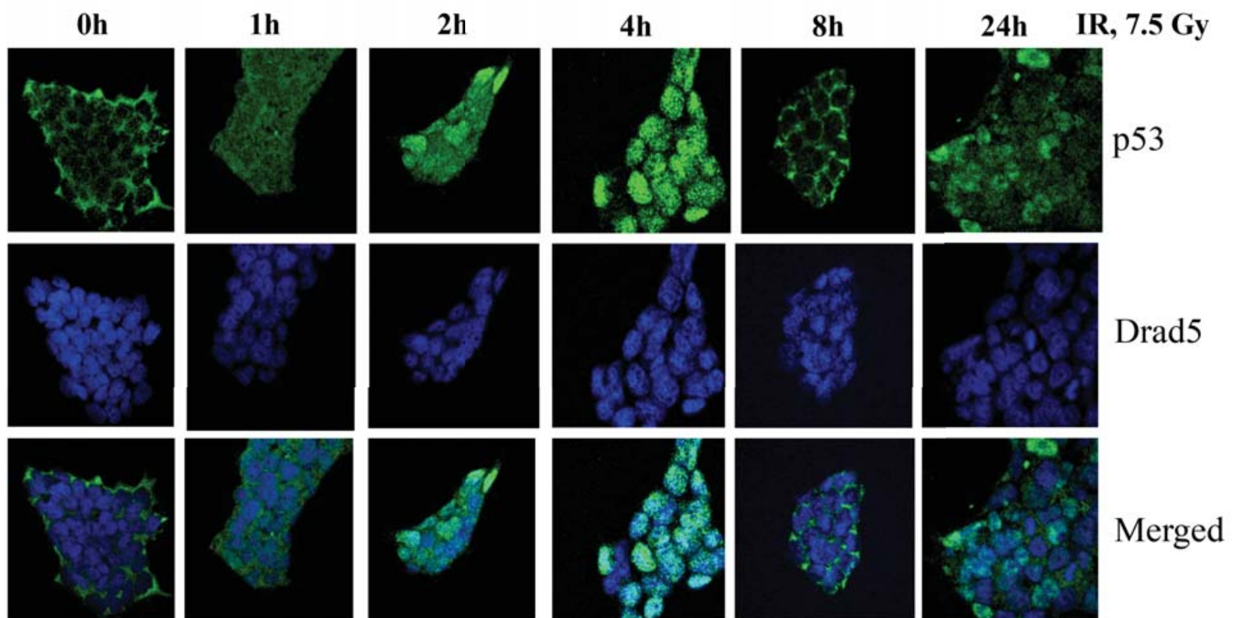

Fig.3.18 P53 accumulated in the nucleus of ES cells after γ-irradiation.

3.18: R1 cells, grown on gelatinized coverslips were exposed to 7.5 Gy of γ-irradiation, harvested after the indicated times and fixed with an ice-cold methanol-acetone (1:1) mix. After blocking, immunostaining was performed using the CM5 (anti-p53) antibody followed by subsequent hybridization with a secondary anti-mouse antibody coupled with Alexa 488 (green) and with Draq5 (blue). After mounting, slides were analyzed by using Carl Zeiss LSM 510 confocal microscope and LSM LSell5 Image Examiner software.

As reported previously (Aladjem et al. 1998; Hong and Stambrook 2004), p53 resided in the cytoplasm of non-irradiated cells. However as early as one hour after irradiation, a significant amount of p53 was detectable in the nucleus or irradiated cells (Fig.3.18) From one to four hours, p53 accumulated in the nucleus to high levels, while at 8 hours after irradiation, p53 was again observed in the cytoplasm of irradiated R1

cells. Interestingly, at 24 hours post irradiation, p53 had changed again its localization. In some cells, it was still cytoplasmic, in others it had accumulated in the nucleus and in some cells, it was present in both cellular compartments (Fig. 3.18).

Since p53 accumulated in the nucleus of irradiated cells, the next question was whether it is also capable of inducing transcription of its target genes. To test this possibility, RNA was prepared from irradiated R1 cells that had been culture twice without feeders (P2) at different time points. For control, RNA was prepared from NIH3T3 cells, from R1 cells that had been culture one without feeders, and from feeder cells. Expression of p53 target genes was then analyzed by qRT-PCR.

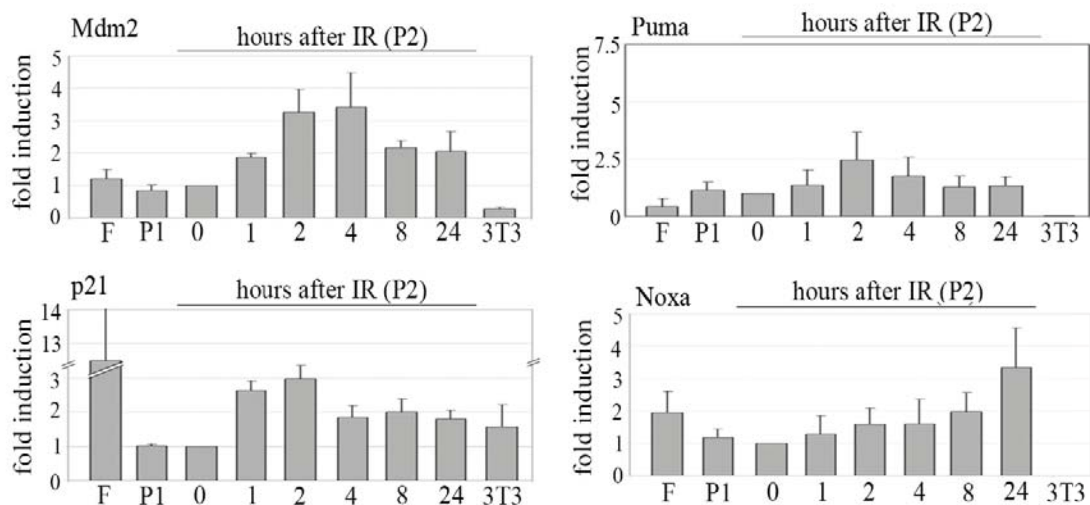

Fig.3.19 Expression of p53 target genes after γ-irradiation

3.19: R1 cells passaged twice on gelatin-coated plates (P2) were irradiated with 7.5 Gray, harvested at the indicated time points, and RNA was prepared For control, RNA was prepared from NIH3T3 cells, from feeder cells and from R1 cells cultured only once with feeders (P1). cDNAs were synthesized and expression of *mdm2, puma, p21* and *noxa* as well as of the housekeeping gene RibPO (34b4) were determined by RT-PCR using gene specific primers. ΔCT values were calculated and the values for the individual p53 target genes were normalised by the values for RibPO. Mean values for the fold induction of the individual transcripts and standard deviations of three independent experiments were plotted. The values for non-irradiated R1 (P2) cells were set to 1.

The results of this experiment are presented on figure 3.19 treatment of cells by ionizing radiation led to the up-regulation of *mdm2*, *p21*, *noxa* and *puma* mRNAs, demonstrating that p53 is transcriptionally active in stem cells.

The next question was, whether the increase in gene expression is also translated into protein. Therefore, R1 cells were irradiated and harvested at different time points. Abundance of the p53 target genes Mdm2, p21, Noxa, Puma, as well as abundance of p53 was then determined by Western Blotting.

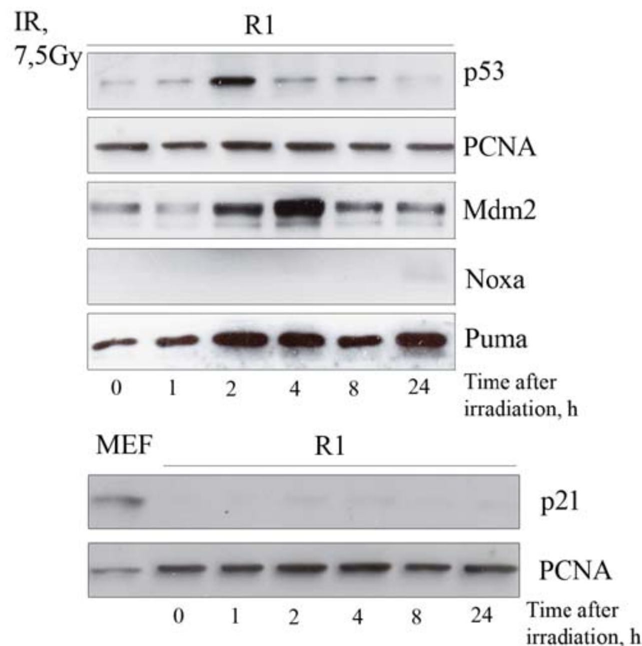

Fig.3.20 Induction of p53 and p53 target genes after γ-irradiation

3.20: R1 cells were γ-irradiated with 7.5 Gray, harvested at the indicated time points and lysed in NP-40 buffer. 50 µg of protein were separated by a 10% SDS-PAGE gel and transferred onto a PVDF membrane. p53 was detected by incubation with the Pab421 antibody, Mdm2 by incubation with the 4B2 antibody, and p21, Noxa, and Puma were detected by incubation of the membrane with protein-specific antibodies. Hybridization with the PC10 (anti-PCNA) antibody was performed for loading control. Western blots were developed by the ECL method.

At one hour post-irradiation, p53 protein levels were transiently increased (Fig. 3.20), followed by induction of the p53 target genes Mdm2 at two to four hours and Puma at two hours. In contrast, an increase in the amount of the Noxa protein was observed at 24 hours, while up-regulation of p21 protein was not remarkable. Thus, induction of Mdm2 and Puma proteins corresponded to the induction of their RNA, while inductions of *p21* and *noxa* genes transcription were not reflected at the protein level.

4. DISCUSSION

4.1 Regulation of p53 abundance in ES cells.

P53 is a crucial tumour suppressor. Recent findings have shown that p53 seems to be one of the key regulators of stem cells (Lin et al. 2005; Meletis et al. 2006; Armesilla-Diaz et al. 2009; Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009). Despite this its regulation and activity in embryonic stem cells needed to be elucidated. In consistency with a previous report (Sabapathy et al. 1997) here it was found that ES cells contain a higher amount of p53 protein than differentiated cells. Since in differentiated cells, the abundance of p53 is mainly regulated by alterations in its stability (Ashcroft and Vousden 1999; Vogelstein et al. 2000), the elevated levels of p53 imply a reduced turnover of this tumour suppressor protein in ES cells. However, when the half-life of p53 was determined of the p53 protein in ES cells, it was found that the p53 protein was even less stable than in differentiated cells (Maltzman and Czyzyk 1984; Blattner et al. 1999). Rapid degradation of p53 may eventually be a mechanism that prevents accumulation of enormous amounts of p53 in ES cells to protect themselves from the anti-proliferative effects of p53.

The elevated protein levels of the p53 protein in ES cells corresponded to a higher amount of p53 RNA, which was, moreover, translated at a higher rate. This observation that ES cells possess a higher amount of p53 RNA is consistent with a previous report which showed that murine foetuses contain a high amount of p53 RNA up to day eleven of their development (Oren 1985; Rogel et al. 1985). Differentiation by treatment with retinoic acid for eight days led to considerable decrease in the rate of p53 translation. At the same time, p53 mRNA exhibited a pro-longed half-life in ES cells in comparison with differentiated derivatives or MEFs. This increased half-life of p53 RNA is certainly one of the reasons for the increased abundance of p53 RNA in ES cells. Whether an increased rate of transcription also contributes to the higher amount of p53 RNA in ES cells was not be determined. In line with the increased stability of p53 RNA in ES cells is the strongly reduced expression of the micro RNAs miRNA-125a and miRNA-125b. These microRNAs have been shown more recently to regulate p53

expression (Le et al. 2009; Zhang et al. 2009). Since microRNAs frequently regulate the stability of target RNAs (Bartel 2004), it is very likely that the absence of miRNA-125a and 125b contribute to the increased stability of p53 RNA in ES cells. Indeed, overexpression of these microRNAs decreased the stability of p53 mRNA in ES cells. MicroRNAs, though, not only reduce the stability of RNAs, they can also affect the rate of translation. The reduced expression of miRNA-125a and miRNA-125b in ES cells may thus also contribute to the increased rate of translation that was observed in ES cells. Therefore, it was not very surprising that overexpression of miRNA-125a and miRNA-125b reduced translation of p53 RNA in ES cells. Together, these results provide a possible mechanism how p53 is maintained at high levels in ES cells. However it is not known as yet what represses expression of miRNA-125a and miRNA-125b in ES cells, and what switches on their expression during differentiation.

In addition to the microRNAs, translation of p53 in ES cells might be regulated by the ribosomal protein L26. L26 binds to the 5'UTR of target mRNAs where it enhances the association with polysomes (Takagi et al. 2005). Although, the level of L26 did not show a clear correlation between ES cells and differentiated cells, overexpression of L26 increased p53 abundance in ES cells. Conversely, downregulation of L26 decreased of p53 levels in ES cells. Surprisingly, overexpression of L26 only increased p53 abundance in ES cells, but not in NIH3T3 cells. This was even more surprising since L26 regulated p53 levels in several human cancer cell line as well as in murine Baf-3 cells (Takagi et al. 2005)}. However, these cell lines are all cancer cell lines and it is currently discussed whether cancer cells might eventually represent a kind of a more undifferentiated cell type (Johnson et al. 2007). Thus it is possible that L26 is particularly or eventually even solely active in undifferentiated or weakly differentiated cells. The reason why overexpression of L26 failed to induce p53 protein in 3T3 cells and eventually in other differentiated cells remains to be determined.

Degradation of p53 occurs within 26S proteasomes in ES cells and it appears as if the same machinery would be used as in differentiated cells. Mdm2 is the most important regulator of p53 in differentiated cells, providing rapid p53 turnover. Here, in this study, it was shown that p53 degradation is also regulated by Mdm2 in ES cells. This is quite surprising since p53 and Mdm2 exist predominantly in different cellular compartments. While p53 is predominantly in the cytoplasm of ES cells, Mdm2 is

located mainly in the nucleus. Despite the localisation in different sub-cellular compartments, Mdm2 co-precipitated with p53 from ES cells. In line with a previous report (Maimets et al. 2008), treatment of ES cells with nutlin, a compound that competes with p53 for binding to Mdm2 (Vassilev et al. 2004), resulted in a strong accumulation of the p53 protein in ES cells. It is presently unclear how nuclear Mdm2 of ES cells can target cytoplasmic p53 for degradation. One possibility is that minor amounts of p53, that are below the detection limit of immunofluorescence microscopy, are present in the nucleus of ES cells were they are ubiquitinated by nuclear Mdm2. Alternatively, some Mdm2 that might be sufficient for p53 ubiquitination, might reside in the cytoplasm of ES cells and from there promote degradation. Why p53 degradation is so efficient in ES cells, although p53 and Mdm2 are spatially separated, is presently unclear. Eventually other ubiquitin ligases for p53 might contribute to p53 degradation in ES cells thus speeding up the process. However, PirH2, which has been shown previously to target p53 for degradation in some cancer cell lines (Leng et al. 2003), appears not to contribute to this process. When we downregulated PirH2, we did not see an increase in p53 abundance, indicating that this E3 ligase does not regulate p53 abundance in ES cells.

P53 degradation is not only regulated by ubiquitin ligases, but also by deubiquitinating enzymes (reviewed by (Boehme and Blattner 2009)). Among this class of proteins is Hausp the most important regulator of p53 that has as yet been identified (Li et al. 2002). Levels of Hausp in ES cells do not differ from that in differentiated cell derivatives. Overexpression of Hausp increased and downregulation of Hausp decreased p53 protein abundance in ES cells, indicating that Hausp in ES cells also regulates p53.

Another enzyme that has been implicated in p53 regulation is Ubc13. Ubc13 is an E2-conguting enzyme, that particularly catalyses the formation of unusual ubiquitin chains (Laine et al. 2006). It is clear now that p53 ubiquitination is not limited only by ubiquitin K48-linkage, which mostly is followed by proteasomal degradation (reviewed in (Lee and Gu)). Ubc13 ubiquitinates p53 via K63-chain (Laine et al. 2006). Ubiquitination of p53 involving Ubc13 attenuates Mdm2-dependent degradation of p53 and leads to cytoplasmic localization of the tumour suppressor protein. In ES cells, downregulation of Ubc13 decreased p53 level, indicating that Ubc13 also takes part in p53 regulation in ES cells.

Taken together, ES cells maintain high level of p53 protein due to increased translation of a higher amount of RNA. Ubiquitinating enzymes including Mdm2 ensure effective degradation of p53 in ES cells despite the activity of Hausp and Ubc13.

.

4.2 p53 resides in the cytoplasm of ES cells

In ES cells, p53 is mainly located in the cytoplasm (Aladjem et al. 1998; Solozobova et al. 2009). Although this is known for several years, neither the principle that anchors p53 in the cytoplasm nor the rational for its cytoplasmic localisation is known as yet. Eventually, ES cells need to respond quickly to changes in their environment, e.g. to cellular stress, which requires a large reservoir of p53 like a "loaded gun". This large amount, though, may require separation from the nucleus where it could activate transcription of pro-apoptotic and cell-cycle arrest inducing genes. Alternatively, ES cells may require a large amount of p53 in the cytoplasm to induce quickly apoptosis by the mitochondrial pathway in response to unfavourable conditions (Mihara r et al. 2003; Erster and Moll 2005; Moll et al. 2005). In this case it is unclear what keeps p53 in the cytoplasm inactive when its activity is unwanted.

Several options are currently discussed that could anchor p53 in the cytoplasm of cells and which may thus also be employed to retain p53 in the cytoplasm of ES cells. Parc and Mortalin were good candidates to keep p53 in the cytoplasm of ES cells. Parc is known t sequester p53 in the cytoplasm of some neuroblastomas, thereby making p53 inactive (Nikolaev et al. 2003). Mortalin is another cytoplasmic p53 anchor in some cancer cells (Wadhwa et al. 2002). It was therefore possible that these proteins may contribute to the cytoplasmic localisation of p53 in ES cells. In contrast to a previous report, Parc was located in the nucleus of both, ES and differentiated cells (Nikolaev et al. 2003). The reason for this discrepancy is unclear. Yet, no correlation of the level or localization of these proteins was found in ES cells and differentiated cells. Co-immunoprecipitation studies also did not reveal n interaction of p53 with Mortalin or Parc. Nevertheless, a clear answer to this question can only be given after downregulation of these factors in ES cells.

Another option that could regulate cytoplasmic localization of p53 in ES cells are post-translational modifications and one of the modifications that have already been

reported to regulate subcellular distribution of p53 is ubiquitination. Ubiquitination is associated with many cellular processes among which proteasomal degradation is certainly to most well-known. However, ubiquitination also regulates nucleosome packaging, endocytic trafficking, nuclear export or lysosomal degradation (Lee and Gu 2010; Haglund and Dikic 2005).

A significant portion of the p53 protein was modified with ubiquitin chains that are linked via lysine 63 in ES cells. Ubiquitination with lysine 63-linked ubiquitin chains has been reported earlier to promote cytoplasmic localization of p53 (Laine et al. 2006). However, the same modification was found in differentiated cells and in ES cells and also in cells after ionizing irradiation, when p53 accumulates in the nucleus of ES cells. Nevertheless, a slight decrease of this modification was observed in irradiated cells and at this condition, p53 becomes nuclear. However, whether ubiquitination of p53 via lysine 63-linked chains is essential for its cytoplasmic localisation or whether another factor may determine its subcellular distribution, remains to be determined.

Lysine 63-linked ubiquitination is frequently implemented by using Ubc13 is an ubiquitin-conjugating enzyme. In fact, it has been shown previously that Ubc13 promotes cytosolic localization of p53 (Laine et al. 2006). Here in this work Ubc13 was found to stabilize p53, however, whether it influences localization of p53 or not, has not as yet been answered.

4.3 p53 is active in ES cells after DNA damage

According to some investigators p53 does not activate G1 ell cycle arrest or apoptosis after DNA damage in ES cells (Aladjem et al. 1998; Hong and Stambrook 2004) while others reported that p53 is activated in response to DNA damage (Sabapathy et al. 1997). It has, moreover, been shown that it induces differentiation of ES cells via repression of *nanog* (Lin et al. 2005) a gene whose expression is characteristic for stem cells. In this work, it was found that p53 is active in resting cells, showing that the p53 protein is also in ES cells in a latent state and can be activated when its activity is required. Moreover, it was found that p53 activates transcription of its target genes in response to DNA damage. After DNA damage, p53 did not activate all, but at least some of its target genes in ES cells. This result supports the findings

from Sabapathy et al., who observed binding of p53 from ES cells to oligonucleotides that correspond to the p53 consensus binding (Sabapathy et al. 1997). Transcription of these genes was facilitated by the presence of p53 in the nucleus of irradiated cells. After IR, p53 accumulated in two waves in the nucleus of ES cells. The first nuclear accumulation occurred at one to two hours after irradiation and correlated with an increase in the amount of the p53 protein. During the second wave of nuclear accumulation of p53 at twenty-four hours after irradiation, an increase in p53 abundance was not seen suggesting that for the second wave of nuclear accumulation p53 was translocated from the cytoplasm into the nucleus. Whether nuclear translocation also contributed to the first wave of nuclear accumulation of p53 is still unclear. Both waves of nuclear accumulation of p53 corresponded to transcriptional activation of target genes. The first wave of nuclear accumulation of p53 correlated with transcriptional activation of *mdm2*, *p21* and *puma* (Nakano and Vousden 2001; Baraket al. 1993; el-Deiry et al. 1994), while *noxa* (Oda, et al. 2000) was transcribed during the second wave of nuclear accumulation of p53. Why some of the p53 target genes are transcribed only during the first wave of nuclear accumulation and why at least one other gene is transcribed only during the second wave of nuclear accumulation is presently unclear. It may be that distinct post-translational modifications of p53 are induced with different kinetics after irradiation and are required for the activation of different p53 target genes (Vousden and Woude 2000).

Experiment with nutlin, compound that inhibits p53 and Mdm2 interaction (Vassilev et al. 2004) was additional evidence that p53 was active in the absence of stress in ES cells since accumulation of p53 led to increase of Mdm2 protein level.

Most surprisingly, p21 was actively transcribed in ES cells and the amount of its RNA was increased further in response to IR, while the protein was hardly detectable before and after irradiation. This result indicates that production of p21 protein is strongly regulated at a post-transcriptional level in ES cells. Eventually, ES cells with damaged DNA require efficient elimination from the population. Since G1 arrest triggered by p21 can prevent cells from S-phase entry and thus from the cellular death program that is executed in the S-phase of cells with damaged DNA, e.g. by the activity of proteins such as Killin, the presence of p21 might counteract this elimination (Polyak et al. 1996; Cho and Liang 2008).

This study clearly shows that p53 can be activated in stem cells despite its

cytoplasmic localization. However, results for p53 activation in stem cells depend on the selection of genes that are analyzed and whether the analysis is performed at the RNA or protein level.

4.4 Conclusion

In this work it was shown that higher levels of p53 in ES cells are maintained on translational level. Lack of microRNA-125a and miRNA-125b, and activity of L26 ribosomal protein, at least partly regulate higher translation of p53 in ES cells. Nevertheless, these cells utilize the same machinery to degrade p53. The reason and mechanism for the cytoplasmic localization of p53 remains unclear.

P53 after DNA damage translocates to the nucleus and is able to activate transcription of its target genes in ES cells.

5. OUTLOOK

The mechanisms of cytoplasmic p53 localization in ES cells and the reason for this in ES cells remain unclear. Despite the abundance and cytoplasmic localization of Mortalin and Parc do not change during differentiation, it cannot be excluded that these proteins do not keep p53 in the cytoplasm of ES cells. However, answer to this question can only be given after downregulation of these factors in ES cells.

Eventually, the p53 protein may interact with other proteins that keep p53 in the cytoplasm of ES cells. Affine chromatography and/or GST-pulldown may identify these proteins interacting with p53 and determining its subcellular localisation in ES cells. After identification of these proteins studying whether they are involved to p53 abundance and localization in ES cells may give an answer to what keeps p53 in the cytoplasm. Also, it may help to find out what is the function of p53 in the cytoplasm of ES cells.

Post-translational modification of p53 as ubiquitination may contribute to the cytoplasmic localization of p53 in ES cells. Ubiquitination of p53 by Ubc13, WWP1 or MSL2 is associated with nuclear export (Laine et al. 2006; Laine and Ronai 2007; Kruse and Gu 2009). Whether the modifications carried out by these proteins regulate p53 localization might be clear after downregulation of Ubc13, WWP1 or MSL2 and checking for p53 subcellular localization.

Another intriguing question is whether downregulation of p53 is a consequence or trigger of differentiation and what are the mechanisms of this downregulation. Downregulation of p53 might answer this question. If downregulation of p53 will not be required for differentiation, this will already be a good indication, that its downregulation is just a consequence.

MiRNA-125a and miRNA-125b had been shown to negatively regulate p53 translation. During differentiation their expression is increased, therefore search for the factor that represses their expression in ES cells might give an answer for p53 regulation during differentiation.

Ribosomal protein L26 positively regulates p53 translation in ES cells. In this work we failed to see that L26 regulates p53 in 3T3 cells. This might be explained that L26 regulates p53 exclusively in undifferentiated cells. Ofir-Rosenfeld and co-workers showed that Mdm2 inhibits regulation of p53 by L26 by targeting it to degradation and

by direct interaction (Ofir-Rosenfeld et al. 2008). Whether this inhibition of L26 by Mdm2 is blocked in ES cells and that happens with this Mdm2-L26 axis during differentiation might be tested.

.

6. REFERENCES

Aasen, T., A. Raya, et al. (2008). "Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes." Nat Biotechnol 26(11): 1276-84.

Abida, W. M., A. Nikolaev, et al. (2007). "FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity." J Biol Chem 282(3): 1797-804.

Aladjem, M. I., B. T. Spike, et al. (1998). "ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage." Curr Biol 8(3): 145-55.

Albrechtsen, N., I. Dornreiter, et al. (1999). "Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53." Oncogene 18(53): 7706-17.

Appella, E. and C. W. Anderson (2001). "Post-translational modifications and activation of p53 by genotoxic stresses." Eur J Biochem 268(10): 2764-72.

Armesilla-Diaz, A., P. Bragado, et al. (2009). "p53 regulates the self-renewal and differentiation of neural precursors." Neuroscience 158(4): 1378-89.

Armesilla-Diaz, A., G. Elvira, et al. (2009). "p53 regulates the proliferation, differentiation and spontaneous transformation of mesenchymal stem cells." Exp Cell Res 315(20): 3598-610.

Ashcroft, M., M. H. Kubbutat, et al. (1999). "Regulation of p53 function and stability by phosphorylation." Mol Cell Biol 19(3): 1751-8.

Ashcroft, M. and K. H. Vousden (1999). "Regulation of p53 stability." Oncogene 18(53): 7637-43.

Banito, A., S. T. Rashid, et al. (2009). "Senescence impairs successful reprogramming to pluripotent stem cells." Genes Dev 23(18): 2134-9.

Barak, Y., T. Juven, et al. (1993). "mdm2 expression is induced by wild type p53 activity." EMBO J 12(2): 461-8.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell 116(2): 281-97.

Bibel, M., J. Richter, et al. (2004). "Differentiation of mouse embryonic stem cells into a defined neuronal lineage." Nat Neurosci 7(9): 1003-9.

Blattner, C., E. Tobiasch, et al. (1999). "DNA damage induced p53 stabilization: no indication for an involvement of p53 phosphorylation." Oncogene 18(9): 1723-32.

Boehme, K. A. and C. Blattner (2009). "Regulation of p53--insights into a complex process." Crit Rev Biochem Mol Biol 44(6): 367-92.

Boheler, K. R., J. Czyz, et al. (2002). "Differentiation of pluripotent embryonic stem cells into cardiomyocytes." Circ Res 91(3): 189-201.

Bonifacino, J. S. (2001). "Metabolic labeling with amino acids." Curr Protoc Mol Biol Chapter 10: Unit 10 18.

Bottger, A., V. Bottger, et al. (1997). "Molecular characterization of the hdm2-p53 interaction." J Mol Biol 269(5): 744-56.

Brehm, A., E. Miska, et al. (1999). "The cell cycle-regulating transcription factors E2F-RB." Br J Cancer 80 Suppl 1: 38-41.

Brooks, C. L. and W. Gu (2003). "Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation." Curr Opin Cell Biol 15(2): 164-71.

Brooks, C. L. and W. Gu (2006). "p53 ubiquitination: Mdm2 and beyond." Mol Cell 21(3): 307-15.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science 282(5393): 1497-501.

Carter, S., O. Bischof, et al. (2007). "C-terminal modifications regulate MDM2 dissociation and nuclear export of p53." Nat Cell Biol 9(4): 428-35.

Chambers, I., D. Colby, et al. (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell 113(5): 643-55.

Chen, D., N. Kon, et al. (2005). "ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor." Cell 121(7): 1071-83.

Chen, J., X. Wu, et al. (1996). "mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein." Mol Cell Biol 16(5): 2445-52.

Chen, L., W. Lu, et al. (1999). "Ubiquitous induction of p53 in tumor cells by antisense inhibition of MDM2 expression." Mol Med 5(1): 21-34.

Cho, Y. J. and P. Liang (2008). "Killin is a p53-regulated nuclear inhibitor of DNA synthesis." Proc Natl Acad Sci U S A 105(14): 5396-401.

Chu, E., S. M. Copur, et al. (1999). "Thymidylate synthase protein and p53 mRNA form an in vivo ribonucleoprotein complex." Mol Cell Biol 19(2): 1582-94.

Chu, H. and E. Reyt (1999). "Association between HPV 16/18 infection and expression of P53 protein in laryngeal papillomas." J Tongji Med Univ 19(4): 275-6.

Chuykin, I. A., M. S. Lianguzova, et al. (2008). "Activation of DNA damage response signaling in mouse embryonic stem cells." Cell Cycle 7(18): 2922-8.

Corbet, S. W., A. R. Clarke, et al. (1999). "P53-dependent and -independent links between DNA-damage, apoptosis and mutation frequency in ES cells." Oncogene 18(8): 1537-44.

Dang, S. M., M. Kyba, et al. (2002). "Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems." Biotechnol Bioeng 78(4): 442-53.

DeLeo, A. B., G. Jay, et al. (1979). "Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse." Proc Natl Acad Sci U S A 76(5): 2420-4.

Donehower, L. A., L. A. Godley, et al. (1995). "Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability." Genes Dev 9(7): 882-95.

Donehower, L. A., M. Harvey, et al. (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature 356(6366): 215-21.

Dornan, D., I. Wertz, et al. (2004). "The ubiquitin ligase COP1 is a critical negative regulator of p53." Nature 429(6987): 86-92.

Dravid, G., Z. Ye, et al. (2005). "Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells." Stem Cells 23(10): 1489-501.

Dulic, V., G. H. Stein, et al. (1998). "Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G2/M-phase transition." Mol Cell Biol 18(1): 546-57.

Dumble, M., L. Moore, et al. (2007). "The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging." Blood 109(4): 1736-42.

Dundas, S. R., L. C. Lawrie, et al. (2005). "Mortalin is over-expressed by colorectal adenocarcinomas and correlates with poor survival." J Pathol 205(1): 74-81.

el-Deiry, W. S., J. W. Harper, et al. (1994). "WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis." Cancer Res 54(5): 1169-74.

Erster, S. and U. M. Moll (2005). "Stress-induced p53 runs a transcription-independent death program." Biochem Biophys Res Commun 331(3): 843-50.

Fang, S., J. P. Jensen, et al. (2000). "Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53." J Biol Chem 275(12): 8945-51.

Filion, T. M., M. Qiao, et al. (2009). "Survival responses of human embryonic stem cells to DNA damage." J Cell Physiol 220(3): 586-92.

Finlay, C. A. (1993). "The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth." Mol Cell Biol 13(1): 301-6.

Freedman, D. A., C. B. Epstein, et al. (1997). "A genetic approach to mapping the p53 binding site in the MDM2 protein." Mol Med 3(4): 248-59.

Frosina, G. (2009). "DNA repair in normal and cancer stem cells, with special reference to the central nervous system." Curr Med Chem 16(7): 854-66.

Gil-Perotin, S., M. Marin-Husstege, et al. (2006). "Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors." J Neurosci 26(4): 1107-16.

Gu, P., D. LeMenuet, et al. (2005). "Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation." Mol Cell Biol 25(19): 8507-19.

Guo, X., W. Ying, et al. (2001). "Proteomic characterization of early-stage differentiation of mouse embryonic stem cells into neural cells induced by all-trans retinoic acid in vitro." Electrophoresis 22(14): 3067-75.

Haglund, K. and I. Dikic (2005). "Ubiquitylation and cell signaling." EMBO J 24(19): 3353-9.

Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature 387(6630): 296-9.

Hermeking, H., C. Lengauer, et al. (1997). "14-3-3 sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell 1(1): 3-11.

Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Annu Rev Biochem 67: 425-79.

Hicke, L. (1997). "Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins." FASEB J 11(14): 1215-26.

Honda, R., H. Tanaka, et al. (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett 420(1): 25-7.

Hong, H., K. Takahashi, et al. (2009). "Suppression of induced pluripotent stem cell generation by the p53-p21 pathway." Nature 460(7259): 1132-5.

Hong, Y., R. B. Cervantes, et al. (2007). "Protecting genomic integrity in somatic cells and embryonic stem cells." Mutat Res 614(1-2): 48-55.

Hong, Y. and P. J. Stambrook (2004). "Restoration of an absent G1 arrest and protection from apoptosis in embryonic stem cells after ionizing radiation." Proc Natl Acad Sci U S A 101(40): 14443-8.

Innocente, S. A., J. L. Abrahamson, et al. (1999). "p53 regulates a G2 checkpoint through cyclin B1." Proc Natl Acad Sci U S A 96(5): 2147-52.

Johnson, D. A., J. Zhang, et al. (2007). "Neuronal differentiation and synaptogenesis in retinoblastoma." Cancer Res 67(6): 2701-11.

Ju, J., J. Pedersen-Lane, et al. (1999). "Regulation of p53 expression by thymidylate synthase." Proc Natl Acad Sci U S A 96(7): 3769-74.

Kastan, M. B., O. Onyekwere, et al. (1991). "Participation of p53 protein in the cellular response to DNA damage." Cancer Res 51(23 Pt 1): 6304-11.

Kaul, S. C., T. Yaguchi, et al. (2003). "Overexpressed mortalin (mot-2)/mthsp70/GRP75 and hTERT cooperate to extend the in vitro lifespan of human fibroblasts." Exp Cell Res 286(1): 96-101.

Kawamura, T., J. Suzuki, et al. (2009). "Linking the p53 tumour suppressor pathway to somatic cell reprogramming." Nature 460(7259): 1140-4.

Keller, G. (2005). "Embryonic stem cell differentiation: emergence of a new era in biology and medicine." Genes Dev 19(10): 1129-55.

Kim, I. and H. Rao (2006). "What's Ub chain linkage got to do with it?" Sci STKE 2006(330): pe18.

Kim, M., A. Habiba, et al. (2009). "Regulation of mouse embryonic stem cell neural differentiation by retinoic acid." Dev Biol 328(2): 456-71.

Kruse, J. P. and W. Gu (2009). "Modes of p53 regulation." Cell 137(4): 609-22.

Kruse, J. P. and W. Gu (2009). "MSL2 promotes Mdm2-independent cytoplasmic localization of p53." J Biol Chem 284(5): 3250-63.

Kubbutat, M. H., S. N. Jones, et al. (1997). "Regulation of p53 stability by Mdm2." Nature 387(6630): 299-303.

Kulikov, R., J. Letienne, et al. "Mdm2 facilitates the association of p53 with the proteasome." Proc Natl Acad Sci U S A.

Kulikov, R., M. Winter, et al. (2006). "Binding of p53 to the central domain of Mdm2 is regulated by phosphorylation." J Biol Chem 281(39): 28575-83.

Laine, A. and Z. Ronai (2007). "Regulation of p53 localization and transcription by the HECT domain E3 ligase WWP1." Oncogene 26(10): 1477-83.

Laine, A., I. Topisirovic, et al. (2006). "Regulation of p53 localization and activity by Ubc13." Mol Cell Biol 26(23): 8901-13.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature 358(6381): 15-6.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature 278(5701): 261-3.

Laptenko, O. and C. Prives (2006). "Transcriptional regulation by p53: one protein, many possibilities." Cell Death Differ 13(6): 951-61.

Le Cam, L., L. K. Linares, et al. (2006). "E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation." Cell 127(4): 775-88.

Le, M. T., C. Teh, et al. (2009). "MicroRNA-125b is a novel negative regulator of p53." Genes Dev 23(7): 862-76.

Lee, J. T. and W. Gu "The multiple levels of regulation by p53 ubiquitination." Cell Death Differ 17(1): 86-92.

Lee, K. H., M. Li, et al. "A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells." Proc Natl Acad Sci U S A 107(1): 69-74.

Lendahl, U., L. B. Zimmerman, et al. (1990). "CNS stem cells express a new class of intermediate filament protein." Cell 60(4): 585-95.

Leng, R. P., Y. Lin, et al. (2003). "Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation." Cell 112(6): 779-91.

Lengner, C. J., H. A. Steinman, et al. (2006). "Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling." J Cell Biol 172(6): 909-21.

Levine, A. J. (1990). "The p53 protein and its interactions with the oncogene products of the small DNA tumor viruses." Virology 177(2): 419-26.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell 88(3): 323-31.

Li, H., M. Collado, et al. (2009). "The Ink4/Arf locus is a barrier for iPS cell reprogramming." Nature 460(7259): 1136-9.

Li, M., C. L. Brooks, et al. (2003). "Mono- versus polyubiquitination: differential control of p53 fate by Mdm2." Science 302(5652): 1972-5.

Li, M., D. Chen, et al. (2002). "Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization." Nature 416(6881): 648-53.

Lin, Q., S. L. Donahue, et al. (2006). "Genome maintenance and mutagenesis in embryonic stem cells." Cell Cycle 5(23): 2710-4.

Lin, T., C. Chao, et al. (2005). "p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression." Nat Cell Biol 7(2): 165-71.

Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." Cell 17(1): 43-52.

Liu, Y., S. E. Elf, et al. (2009). "p53 regulates hematopoietic stem cell quiescence." Cell Stem Cell 4(1): 37-48.

Loh, Y. H., Q. Wu, et al. (2006). "The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells." Nat Genet 38(4): 431-40.

Lou, M. A., S. L. Tseng, et al. (1997). "Novel patterns of p53 abnormality in breast cancer from Taiwan: experience from a low-incidence area." Br J Cancer 75(5): 746-51.

Lowry, W. E., L. Richter, et al. (2008). "Generation of human induced pluripotent stem cells from dermal fibroblasts." Proc Natl Acad Sci U S A 105(8): 2883-8.

Maimets, T., I. Neganova, et al. (2008). "Activation of p53 by nutlin leads to rapid differentiation of human embryonic stem cells." Oncogene 27(40): 5277-87.

Maki, C. G., J. M. Huibregtse, et al. (1996). "In vivo ubiquitination and proteasome-mediated degradation of p53(1)." Cancer Res 56(11): 2649-54.

Malkin, D., F. P. Li, et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." Science 250(4985): 1233-8.

Maltsev, V. A., A. M. Wobus, et al. (1994). "Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents." Circ Res 75(2): 233-44.

Maltzman, W. and L. Czyzyk (1984). "UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells." Mol Cell Biol 4(9): 1689-94.

Marchenko, N. D. and U. M. Moll (2007). "The role of ubiquitination in the direct mitochondrial death program of p53." Cell Cycle 6(14): 1718-23.

Marchenko, N. D., S. Wolff, et al. (2007). "Monoubiquitylation promotes mitochondrial p53 translocation." EMBO J 26(4): 923-34.

Marion, R. M., K. Strati, et al. (2009). "A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity." Nature 460(7259): 1149-53.

Maynard, S., A. M. Swistowska, et al. (2008). "Human embryonic stem cells have enhanced repair of multiple forms of DNA damage." Stem Cells 26(9): 2266-74.

Mazan-Mamczarz, K., S. Galban, et al. (2003). "RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation." Proc Natl Acad Sci U S A 100(14): 8354-9.

Melchior, F. and L. Hengst (2002). "SUMO-1 and p53." Cell Cycle 1(4): 245-9.

Meletis, K., V. Wirta, et al. (2006). "p53 suppresses the self-renewal of adult neural stem cells." Development 133(2): 363-9.

Michael, D. and M. Oren (2003). "The p53-Mdm2 module and the ubiquitin system." Semin Cancer Biol 13(1): 49-58.

Midgley, C. A. and D. P. Lane (1997). "p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding." Oncogene 15(10): 1179-89.

Mihara, M., S. Erster, et al. (2003). "p53 has a direct apoptogenic role at the mitochondria." Mol Cell 11(3): 577-90.

Mitsui, K., Y. Tokuzawa, et al. (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." Cell 113(5): 631-42.

Molchadsky, A., I. Shats, et al. (2008). "p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner." PLoS One 3(11): e3707.

Moll, U. M., A. G. Ostermeyer, et al. (1996). "Cytoplasmic sequestration of wild-type p53 protein impairs the G1 checkpoint after DNA damage." Mol Cell Biol 16(3): 1126-37.

Moll, U. M., S. Wolff, et al. (2005). "Transcription-independent pro-apoptotic functions of p53." Curr Opin Cell Biol 17(6): 631-6.

Momand, J., G. P. Zambetti, et al. (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell 69(7): 1237-45.

Nagao, M., K. Campbell, et al. (2008). "Coordinated control of self-renewal and differentiation of neural stem cells by Myc and the p19ARF-p53 pathway." J Cell Biol 183(7): 1243-57.

Nakagawa, M., M. Koyanagi, et al. (2008). "Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts." Nat Biotechnol 26(1): 101-6.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell 7(3): 683-94.

Nichols, J., B. Zevnik, et al. (1998). "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." Cell 95(3): 379-91.

Nikolaev, A. Y., M. Li, et al. (2003). "Parc: a cytoplasmic anchor for p53." Cell 112(1): 29-40.

O'Neill, L. A. (2009). "Regulation of signaling by non-degradative ubiquitination." J Biol Chem 284(13): 8209.

Oda, E., R. Ohki, et al. (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science 288(5468): 1053-8.

Ofir-Rosenfeld, Y., K. Boggs, et al. (2008). "Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26." Mol Cell 32(2): 180-9.

Ogawa, K., R. Nishinakamura, et al. (2006). "Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells." Biochem Biophys Res Commun 343(1): 159-66.

Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature 362(6423): 857-60.

Oren, M. (1985). "The p53 cellular tumor antigen: gene structure, expression and protein properties." Biochim Biophys Acta 823(1): 67-78.

Park, I. H., P. H. Lerou, et al. (2008). "Generation of human-induced pluripotent stem cells." Nat Protoc 3(7): 1180-6.

Park, M., H. D. Chae, et al. (2000). "Constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated G2-M arrest." Cancer Res 60(3): 542-5.

Park, Y. and S. L. Gerson (2005). "DNA repair defects in stem cell function and aging." Annu Rev Med 56: 495-508.

Pesce, M., X. Wang, et al. (1998). "Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation." Mech Dev 71(1-2): 89-98.

Picksley, S. M. and D. P. Lane (1993). "The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53?" Bioessays 15(10): 689-90.

Polyak, K., T. Waldman, et al. (1996). "Genetic determinants of p53-induced apoptosis and growth arrest." Genes Dev 10(15): 1945-52.

Qin, H., T. Yu, et al. (2007). "Regulation of apoptosis and differentiation by p53 in human embryonic stem cells." J Biol Chem 282(8): 5842-52.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature 414(6859): 105-11.

Rogel, A., M. Popliker, et al. (1985). "p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors." Mol Cell Biol 5(10): 2851-5.

Rolletschek, A., H. Chang, et al. (2001). "Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors." Mech Dev 105(1-2): 93-104.

Sabapathy, K., M. Klemm, et al. (1997). "Regulation of ES cell differentiation by functional and conformational modulation of p53." EMBO J 16(20): 6217-29.

Saretzki, G., L. Armstrong, et al. (2004). "Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells." Stem Cells 22(6): 962-71.

Saretzki, G., T. Walter, et al. (2008). "Downregulation of multiple stress defense mechanisms during differentiation of human embryonic stem cells." Stem Cells 26(2): 455-64.

Schlamp, C. L., G. L. Poulsen, et al. (1997). "Nuclear exclusion of wild-type p53 in immortalized human retinoblastoma cells." J Natl Cancer Inst 89(20): 1530-6.

Sharpless, N. E. (2005). "INK4a/ARF: a multifunctional tumor suppressor locus." Mutat Res 576(1-2): 22-38.

Shieh, S. Y., J. Ahn, et al. (2000). "The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites." Genes Dev 14(3): 289-300.

Shieh, S. Y., M. Ikeda, et al. (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell 91(3): 325-34.

Smits, V. A., R. Klompmaker, et al. (2000). "p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint." J Biol Chem 275(39): 30638-43.

Solozobova, V., A. Rolletschek, et al. (2009). "Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage." BMC Cell Biol 10: 46.

Stommel, J. M., N. D. Marchenko, et al. (1999). "A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking." EMBO J 18(6): 1660-72.

Sun, X. F., J. M. Carstensen, et al. (1992). "Prognostic significance of cytoplasmic p53 oncoprotein in colorectal adenocarcinoma." Lancet 340(8832): 1369-73.

Takagi, M., M. J. Absalon, et al. (2005). "Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin." Cell 123(1): 49-63.

Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell 131(5): 861-72.

Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell 126(4): 663-76.

Taylor, W. R., S. E. DePrimo, et al. (1999). "Mechanisms of G2 arrest in response to overexpression of p53." Mol Biol Cell 10(11): 3607-22.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene 20(15): 1803-15.

Tichy, E. D. and P. J. Stambrook (2008). "DNA repair in murine embryonic stem cells and differentiated cells." Exp Cell Res 314(9): 1929-36.

Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." Nat Rev Cancer 6(12): 909-23.

Tyner, S. D., S. Venkatachalam, et al. (2002). "p53 mutant mice that display early ageing-associated phenotypes." Nature 415(6867): 45-53.

Ueda, H., S. J. Ullrich, et al. (1995). "Functional inactivation but not structural mutation of p53 causes liver cancer." Nat Genet 9(1): 41-7.

Utikal, J., J. M. Polo, et al. (2009). "Immortalization eliminates a roadblock during cellular reprogramming into iPS cells." Nature 460(7259): 1145-8.

Van Sloun, P. P., J. G. Jansen, et al. (1999). "The role of nucleotide excision repair in protecting embryonic stem cells from genotoxic effects of UV-induced DNA damage." Nucleic Acids Res 27(16): 3276-82.

Varkonyi-Gasic, E., R. Wu, et al. (2007). "Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs." Plant Methods 3: 12.

Vassilev, L. T., B. T. Vu, et al. (2004). "In vivo activation of the p53 pathway by small-molecule antagonists of MDM2." Science 303(5659): 844-8.

Vogelstein, B., D. Lane, et al. (2000). "Surfing the p53 network." Nature 408(6810): 307-10.

Vousden, K. H. and D. P. Lane (2007). "p53 in health and disease." Nat Rev Mol Cell Biol 8(4): 275-83.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer 2(8): 594-604.

Vousden, K. H. and C. Prives (2009). "Blinded by the Light: The Growing Complexity of p53." Cell 137(3): 413-31.

Vousden, K. H. and G. F. Woude (2000). "The ins and outs of p53." Nat Cell Biol 2(10): E178-80.

Wadhwa, R., H. Ando, et al. (2003). "Targeting mortalin using conventional and RNA-helicase-coupled hammerhead ribozymes." EMBO Rep 4(6): 595-601.

Wadhwa, R., K. Taira, et al. (2002). "An Hsp70 family chaperone, mortalin/mthsp70/PBP74/Grp75: what, when, and where?" Cell Stress Chaperones 7(3): 309-16.

Wadhwa, R., K. Taira, et al. (2002). "Mortalin: a potential candidate for biotechnology and biomedicine." Histol Histopathol 17(4): 1173-7.

Wang, X., H. Y. Kua, et al. (2006). "p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling." J Cell Biol 172(1): 115-25.

Wernig, M., A. Meissner, et al. (2007). "In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state." Nature 448(7151): 318-24.

Wu, L. and A. J. Levine (1997). "Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene." Mol Med 3(7): 441-51.

Xirodimas, D. P., M. K. Saville, et al. (2004). "Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity." Cell 118(1): 83-97.

Xirodimas, D. P., C. W. Stephen, et al. (2001). "Cocompartmentalization of p53 and Mdm2 is a major determinant for Mdm2-mediated degradation of p53." Exp Cell Res 270(1): 66-77.

Yu, J., M. A. Vodyanik, et al. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science 318(5858): 1917-20.

Yu, Z. K., R. K. Geyer, et al. (2000). "MDM2-dependent ubiquitination of nuclear and cytoplasmic P53." Oncogene 19(51): 5892-7.

Zhan, Q., M. J. Antinore, et al. (1999). "Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45." Oncogene 18(18): 2892-900.

Zhang, Y., J. S. Gao, et al. (2009). "MicroRNA 125a and its regulation of the p53 tumor suppressor gene." FEBS Lett 583(22): 3725-30.

Zhang, Y. and Y. Xiong (2001). "Control of p53 ubiquitination and nuclear export by MDM2 and ARF." Cell Growth Differ 12(4): 175-86.

Zhao, Y., X. Yin, et al. (2008). "Two supporting factors greatly improve the efficiency of human iPSC generation." Cell Stem Cell 3(5): 475-9.

Curriculum vitae

NAME: Valeriya Solozobova

ADRRESS: Rueppurrer str. 106, 76137 Karlsruhe

E-mail : valeriya.solozobova@kit.edu

Place and Date of Birth: Moscow, Russia, December 4, 1981

FIELDS OF SPECIALIZATION: biochemistry, molecular biology

BUSINESS ADDRESS: Institute of Toxicology and Genetics, KIT, Campus Nord, 76344 Eggenstein-Leopoldshafen, Hermann-von-Helmholtz-Platz-1.

CONTACT TELEPHONE: 0049-7221-82-2714

Educational Background:

1999 – Salakhov Higher Grammar School - Laboratory of Tyumen Region, Surgut, Russia, Silver Medal Winner.

1999 – 2004: Graduate Student, Kazan State University, Department of Biochemistry, Kazan, Russia. Diploma with Honors.

2004 – June 2007: Post-Graduate Student, Institute of Cell Biophysics, Pushchino, Russia.

2007 - 2010: PhD Student, Insitute of Toxicolgy and Genetics, Karslruhe Institute of Technology, Campus Nord, Germany

Languages: English (fluent), Russian (native), German (reading and understanding using a dictionary).

Publication list:

Publications:

Solozobova V, Rolletschek A, Blattner C. (2009) Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. BMC Cell Biology 10:46

Solozobova V, Blattner. C. Regulation of p53 in embryonic stem cells. (submitted after revision for Exp. Cell Research)

Poster presentation:

Solozobova V, Blattner C. Regulation of p53 (EMBL conference on Stem cells, Tissue Homeostasis and Cancer (12-15.05.2010)

Acknowledgements.

First of all I would like to thank Dr. Christine Blattner, for being such a good supervisor, for cheering me up and putting me down when needed, for the many criticisms that helped to improve my project.

I'd also like to thank the former and the present members of the lab 203 for the nice time we spent together. Especially I thanks a lot Roman.

I would like also to thank all members of ITG for a lot of help to me to handle some equipment, and also, in general, for being such a nice collective.

My life would have been harder without the support of some special persons I met during my experience in Germany.

Thanks to Dieter for his understanding of my long work hours and his supporting and his advises.

Thanks to my friends Danilo, Misha, Michael, Christian, Viviene, Marika, Justin, Julia, Flo, and Irene.

I would like to thank my parents, my brother and my grandparents for being close to me and for the feeling of constant support despite the long distance between us.