# Regulation of p53 by Fam83F
# “family with sequence similarity 83F”

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DISSERTATION

von

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Mohammed Salama

Karlsruhe, 08.01.2018

# Zusammenfassung

p53 ist eines der wichtigsten Tumorsuppressor-Proteine. Die häufigsten biologischen Konsequenzen der p53-Aktivierung sind Zellzyklus-Arrest, Induktion von Apoptose und zelluläre Seneszenz. In der Zelle wird p53 hauptsächlich durch das Mdm2 Protein, eine Ubiquitin Ligase die p53 dem Abbau in zellulären Proteasomen zuführt, kontrolliert.

Obwohl p53 in etwa der Hälfte der menschlichen Tumore durch Mutation inaktiviert ist, ist es wegen seiner hohen anti-proliferativen Aktivität für die übrigen Fälle als mögliches Therapeutikum interessant. Um p53 für eine therapeutische Anwendung benutzen zu können, ist es notwendig zu wissen, wie p53 reguliert wird.

In unserem Labor wurde Fam83F als neuer Regulator von p53 identifiziert. Für meine Doktorarbeit untersuchte ich den molekularen Mechanismus, mit dem Fam83F p53 reguliert.

Ich konnte zeigen, dass Fam83F p53 in einer proteasom-abhängigen Weise durch Verringerung der p53-Ubiquitinierung stabilisiert. Fam83F interagiert mit dem C-Terminus von p53, der die Haupt-Ubiquitinierungsstellen enthält. Die durch Fam83F verursachte Zunahme der p53 Menge spiegelte sich auch in einer Zunahme der p53-Aktivität wider. Als Reaktion auf DNA-Schäden wurde Fam83F induziert, was bereits darauf hindeutete, dass Fam83F zur Aktivierung von p53 nach DNA-Schäden beitragen könnte. Entsprechend war die Expression von p21 nach einer Schädigung der DNA in Zellen verringert, die mit Lentiviren infiziert worden waren welch für eine gegen Fam83F gerichtete shRNA kodierten. Die gleichen Lentiviren erhöhten auch die Zellproliferation. Fam83F ko-präzipitierte zwar auch mit mutiertem p53, führte aber nicht zu einer Mengenzunahme von mutiertem p53 Protein.

# ABSTRACT

p53 is one of the most important tumor suppressor proteins. The most common biological consequences of p53 activation are cell cycle arrest, induction of apoptosis and cellular senescence. In the cell, p53 protein levels are kept under tight control by its negative regulator Mdm2, an ubiquitin ligase that targets p53 for degradation.

Because of its anti-proliferative activity, p53 could potentially be used for cancer therapy. It is therefore important to determine how p53 is regulated. In our lab, we identified Fam83F as a novel regulator of p53 by performing a mammalian cell culture overexpression screen using a cDNA library from the teleost Medaka. For my PhD, I have investigated the molecular mechanism by which Fam83F regulates p53.

My Data show that Fam83F stabilizes p53 in a proteasome-dependent manner, by reducing p53 ubiquitination. Fam83F interacts with the C-terminus of p53, which contains the main ubiquitination sites. The increase in p53 abundance caused by Fam83F was also reflected by an increase in p53 activity. Fam83F is induced in response to DNA damage, suggesting it may contribute to the activation of p53 under these conditions. Indeed, knock-down of Fam83F in RKO cells reduced p21 expression and increased the rate of cell proliferation. Fam83F also interacts with mutant p53 but does not increase the protein levels of mutant p53.

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# 1. Introduction

p53 was discovered in 1979 where it was found to co-precipitate with the large T-antigen of simian virus 40 (SV40) in extracts of SV40-transformed cells (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). At the same time, it was shown that cellular p53 levels were high in cells with functional T-antigen, but much lower in cells with mutated, non-functional T-antigen (Linzer et al., 1979). Soon thereafter, it was found that transformed cell lines expressed high levels of p53 in comparison to non-transformed cells (DeLeo et al., 1979). A few years later, it was shown that co-transfection of a plasmid expressing p53 and of a plasmid encoding the oncogene ras led to cell transformation (Eliyahu et al., 1984; Parada et al., 1984). Even p53 on its own was found to immortalize early passage rat chondrocytes (Jenkins et al., 1984). Because of the tumor promoting activities p53 was initially regarded as an oncogene (Jenkins et al., 1985) and it took several years to recognize that p53 is actually not an oncogene but a tumor suppressor protein. In these years several observations were made that finally led to the idea that p53 might be a tumor suppressor protein and not an oncogene. The first clue came from David Wolf and Varda Rotter, who found that p53 was inactivated by a retroviral insertion into the p53 gene in an Abelson-murine-leukemia-virus-transformed cell line (Wolf and Rotter, 1984; Wolf et al., 1984). Similar observations were reported in groups studying leukemia that were induced by the Friend erythroleukemia virus (Mowat et al., 1985; Ben David et al., 1988). It was furthermore reported that the p53 coding sequence was deleted in the human leukemia-derived cell line HL60 (Wolf and Rotter, 1985). These observations indicated that loosing p53 can promote cancer. However, at that time, these findings were considered as exceptional cases and their importance was not realized. In 1989, a new cDNA clone was investigated in the laboratory of Arnold Levine and with this clone it was not possible to reproduce the transforming effects that were observed with earlier clones of *p53*. The mystery was solved when the sequence of the novel cDNA clone of *p53* and the sequence of the earlier clones were compared and it emerged that the sequences of the clones were not identical. This result suggested that at least some, if not all, of the clones carried

mutations in the p53 coding region (Finlay et al., 1989). This suggestion was approved when the sequence of wild-type p53 (wt p53) that was derived from normal, untransformed tissue, was established (Eliyahu et al., 1988; Finlay et al., 1988). Subsequent experiments showed that p53 is indeed a tumor suppressor protein and that the cDNAs that were initially isolated were mutant forms of p53 (Baker et al., 1989).

## 1.1 p53: "Guardian of the genome"

Genome integrity is based on equal segregation of faithfully repaired and replicated chromosomes to daughter cells during cell division. p53 controls these steps and thus maintains genomic integrity. Therefore, p53 is also called "guardian of the genome" (Lane, 1992). Upon DNA damage, p53 accumulates and induces cell cycle arrest at the restriction point at the end of the G1 phase (Kastan et al., 1991). This arrest of the cell cycle allows time to the cell to repair the DNA lesion prior to the next cell division. If the lesion cannot be repaired, p53 might also trigger cell suicide by apoptosis (Figure 1.1; Yonish-Rouach et al., 1991; Lane, 1992).

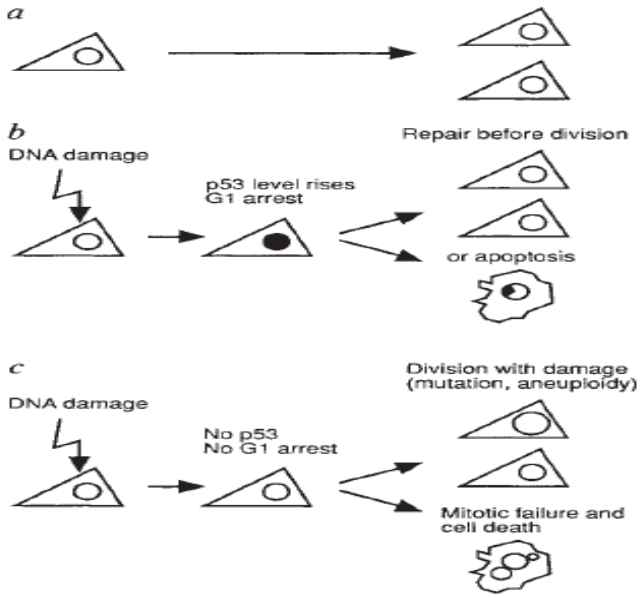


**Figure 1.1: p53 as a "guardian of the genome". (a)** p53 is not required during cell division. **(b)** In response to DNA damage, p53 is induced and initiates DNA repair or apoptosis. **(c)** Cells with mutant p53 replicate damaged DNA resulting in mutations and aneuploidy. (From: Lane, 1992).

How important the guardian of the genome function is displayed in several human and mouse cancer models that show that the increase in tetraploid and polyploidy tumor cells is correlated with loss of p53 function (Galipeau et al., 1995; Ramel et al., 1995). Moreover, p53-null mice frequently develop thymic lymphoma (Donehower et al., 1992). Sarcomas and lymphomas from p53+/- mice showed more chromosomal instability than tumors with two wild-type alleles (Venkatachalam et al., 1998). In addition, cells that possess wild-type p53 but are deficient in p53 transcriptional targets such as *p21* or *gadd45* (growth arrest and DNA damage-inducible protein) also accumulate aberrant chromosomal numbers (Fukasawa et al., 1997; Hollander et al., 1999; Mantel et al., 1999). Therefore, p53 is considered to be also "a guardian of ploidy" (Aylon and Oren, 2011).

## 1.2 The structure of p53

p53 is a nuclear phosphoprotein that is encoded by a twenty-kilobases-spanning gene located on chromosome 17. The gene contains 10 introns and 11 exons (Isobe et al., 1986; Lamb and Crawford, 1986). The human p53 protein consists of 393 amino acids that are divided into several functional and structural domains (Figure 1.2). Residues 1-42 represent the N-terminus that contains the transactivation domain. This N-terminus is followed by a proline rich region that encompasses residues 61-94. Residues 102-292 represent the central core domain that contains the DNA binding domain, and residues 301-393 represent the C-terminal region that holds the oligomerization domain (residues 324-355) and a basic carboxyterminal domain (residues 363-393). The C-terminal region also contains a nuclear export signal (NES) and a nuclear localization signal (NLS) that facilitate the shuttling of p53 between the nucleus and the cytoplasm (Wang et al., 1992; Liang and Clarke, 1999; Stommel et al., 1999).

The N-terminus of p53 is required for its transactivation activity (Lin et al., 1994). Mdm2 (murine double minute), a negative regulator of p53, associates with this N-terminus of p53 (Oliner et al., 1993; Kussie et al., 1996). Mapping of the Mdm2-

binding site on p53 showed the involvement of residues 18-26 of p53 in the interaction with Mdm2 (Böttger et al., 1997). Site-directed mutagenesis showed that residues Phe19, Trp23 and Leu26 are the most critical sites for the interaction (Böttger et al., 1997). Deletion of this Mdm2-binding site on p53 resulted in resistance to Mdm2-mediated degradation of p53 (Moll and Petrenko, 2003). The N-terminus of p53 furthermore associates with p300/CBP (cAMP-response element binding protein (CREB)-Binding protein, Jenkins et al., 2009). p300/CBP, p53 and PML (promyelocytic leukemia protein) form a ternary complex in nuclear bodies that is required for p53 acetylation (Pearson et al., 2000). Phosphorylation of Ser15 in the N-terminus of p53 increased the recruitment of p300/CBP (Lambert et al., 1998) resulting in enhanced acetylation of lysine residues in the C-terminus of p53. This acetylation then leads to an increase in sequence-specific binding of p53 to DNA (Gu and Roeder, 1997).

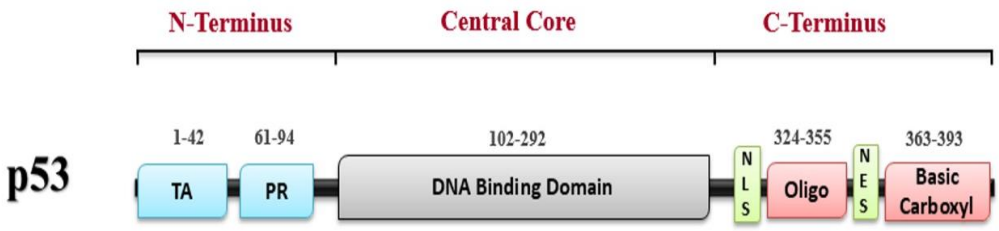


**Figure 1.2: Structure of the p53 protein.** The p53 protein contains 393 amino acids and is divided into several functional and structural domains. The N-terminus contains the transactivation domain (TA, residues 1-42) followed by a proline-rich region (PR; residues 61-94). The central core domain contains the DNA binding domain (residues 102-292). The C- terminal region contains the nuclear localization signal (NLS) followed by the oligomerization domain (Oligo, residues 324-355), the nuclear export signal (NES) and a basic carboxyterminal domain (residues 363-393).

The N-terminus of p53 is followed by a proline-rich domain, a rigid linker that favors a polyproline II helix structure that keeps the transactivation domain of p53 away from the DNA binding domain (Wells et al., 2008). The DNA-binding domain is required for sequence-specific DNA binding. The consensus sequence of of p53 consists of two copies of the 10-bp motif: 5'PuPuPuC(A/T)-(T/A)GPyPyPy- 3'

(Kern et al., 1991). Most p53 cancer mutations are located in the DNA binding domain (Joerger and Fersht, 2007). Particularly frequent are mutations found at residues Arg175, Gly245, Arg248, Arf249, Arg273, and Arg282 (Joerger et al., 2006). The oncogenic mutations frequently decrease the stability of the thermodynamically unstable DNA-binding domain causing its rapid unfolding at body temperature (Friedler et al., 2003; Butler and Loh, 2006).

The C-terminus of p53 plays a pivotal role in the sequence-specific DNA binding (Hupp et al., 1992). The C-terminus of p53 interferes with the binding of the DNA-binding domain to the response elements and therefore functions as a negative regulatory domain (Anderson et al., 1997; Ahn and Prives, 2001; Vousden, 2002). Residues 80-93 of the proline-rich domain in the N-terminus of p53 furthermore associate with the C-terminus and thereby function as an additional negative regulatory domain of p53 (Muller-Tiemann et al., 1998). The C-terminus of p53 furthermore contains the oligomerization domain, a domain that is responsible for the tetramerization of p53 (Jeffrey et al., 1995). In contrast to other transcription factors that oligomerize upon binding of ligands or upon association with DNA, p53 is already present as a tetramer prior to its association with DNA (Friedman et al., 1993). The oligomerization domain consists of a short β-strand and α-helix. Through the process of intermolecular α-helix packing and β-sheet formation assemble four monomers into a tightly packed p53 tetramer that binds to DNA very efficiently (Clore et al., 1994; Jeffrey et al., 1995). The tetramerization of p53 is furthermore essential for its C-terminal acetylation (Itahana et al., 2008). The basic carboxyterminal domain of p53 furthermore contains the main acetylation sites including Lys370, Lys372, Lys373, Lys381 and Lys382 (Gu and Roeder, 1997). These acetylation sites also serve as the main ubiquitination sites for p53 (Chan et al., 2006) and therefore also control the stability of the p53 protein (Lohrum et al., 2001; Li et al., 2002).

## 1.3 The regulation of p53 abundance and activity

The p53 protein is under tight regulation that is achieved by a network of positive and negative regulators. The levels of regulation are by (i) protein stability, (ii) protein activity and (iii) subcellular distribution (Jimenez et al., 1999; Meek, 1999; Prives and Hall, 1999; Ljungman, 2000). Posttranslational modifications (PTMs) play particular roles in the regulation of p53. Approximately fifty amino acids of p53 are subjected to PTMs. These PTMs include ubiquitination, sumoylation, phosphorylation, acetylation, methylation, glycosylation, neddylation and poly-ribosylation (Boehme and Blattner, 2009).

### 1.3.1 Regulation of p53 abundance

P53 is present at very low amounts during normal cell proliferation. These low levels are due to rapid degradation after protein synthesis (Kubbutat and Vousden, 1998). Degradation of p53 occurs in 26S proteasomes and requires prior ubiquitination (Fang et al., 2000). Ubiquitination of proteins is performed by three classes of

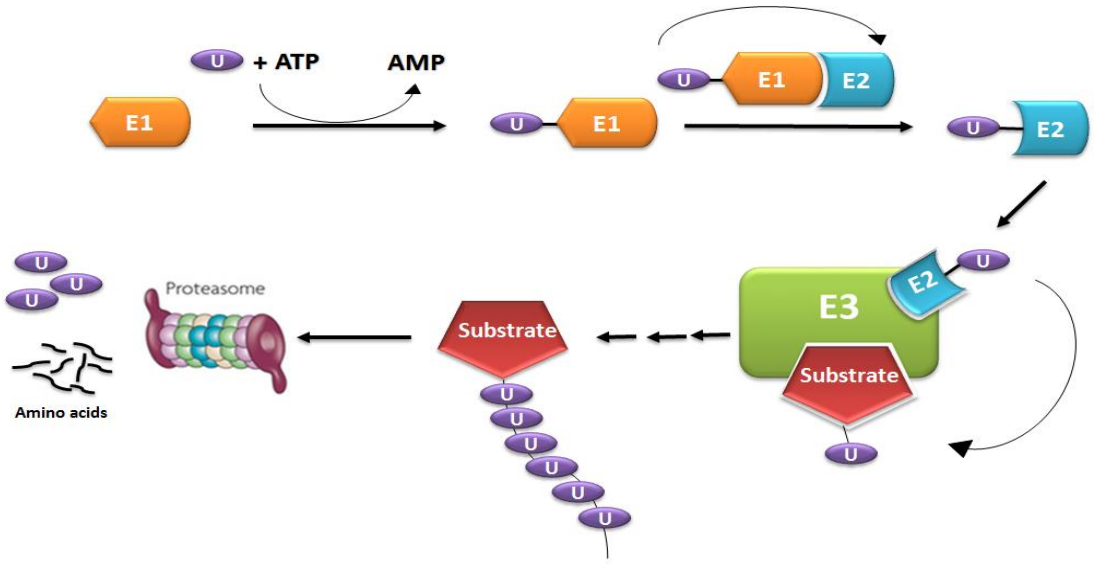


**Figure 1.3: The ubiquitination cycle.** Ubiquitination and degradation of proteins in 26S proteasomes occur through a sequential process involving three classes of enzymes: The E2 protein (ubiquitin activating enzyme) activates the ubiquitin molecule that is then conjugated to an E2 protein (ubiquitin conjugating enzyme). The E3 enzyme (ubiquitin ligase) recognizes a specific substrate protein and catalyzes the transfer of ubiquitin onto the target protein.

enzymes; an E1 enzyme (ubiquitin activating protein), an E2 enzyme (ubiquitin conjugating enzyme) and an E3 enzyme (ubiquitin ligase; Figure 1.3, Varshavsky, 1997). The first step of ubiquitination is an ubiquitin-activating step. By utilizing ATP the E1 enzyme catalyzes the formation of ubiquitin adenylate. This intermediate then forms a high-energy thioester blond with a cysteine in the E1 enzyme. Thereafter, the ubiquitin is transferred to a cysteine on an E2 enzyme. In the final step the E3 enzyme mediates the formation of an isopeptide bond between the C-terminus of ubiquitin and a ε-amino group of a lysine residue in the target protein (Glickman and Ciechanover, 2002). The E3 enzyme plays the main role in the recognition of substrates (Hershko and Ciechanover, 1992).

Amongst the ten E3 proteins that are reported to control p53 abundance is the RING domain ubiquitin ligase Mdm2 of highest importance for p53 (Boehme and Blattner, 2009). Mdm2 is, for instance, essential for preventing p53 activation during embryonic development. Mice with a genetic deletion of both Mdm2 alleles die during embryogenesis, but they survive when they also lack the *p53* gene (Jones et al., 1995; Montes de Oca-Luna et al., 1995). The *mdm2* gene (Figure 1.4) was initially discovered on a double minute chromosome of spontaneously transformed mouse 3T3 fibroblasts (Cahilly-Snyder et al., 1987).


**Figure 1.4: Structure of the Mdm2 protein.** The drawing shows the full-length Mdm2 protein. Known motifs are indicated. The p53 interaction domain is located in the amino terminal domain. NLS: nuclear localization signal; NES: nuclear export signal; zn-finger: zinc-finger domain; NoLS: nuclear localization signal; RING: (really interesting new gene) finger domain has an ubiquitin ligase domain (modified from: Iwakuma and Lozano, 2003).

Soon after its identification, Mdm2 was found to interact with p53 (Momand et al., 1992) and a couple of years later, it was found that Mdm2 possesses ubiquitin ligase towards p53 (Figure 1.5, Honda and Yasuda, 2000). Importantly, Mdm2 itself is a target gene of p53 (Barak et al., 1993; Juven et al., 1993). Thus, p53 and Mdm2 are connected to each other through a negative feedback loop that ensures that cellular p53 levels are kept low in the absence of stress (Picksley and Lane, 1993).

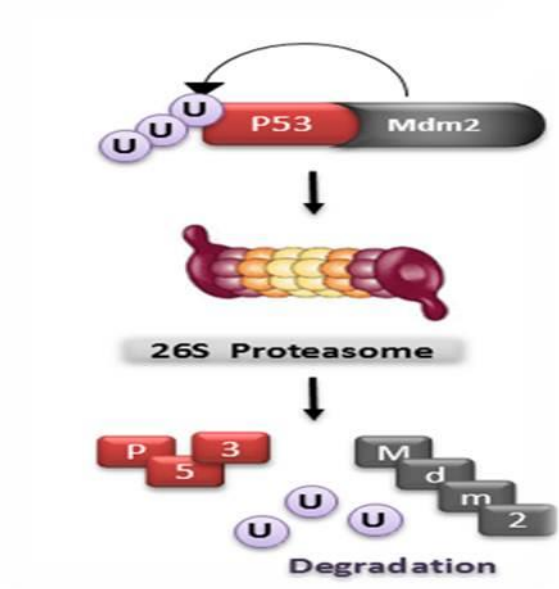


**Figure 1.5: Control of p53 stability by Mdm2.** During normal cellular conditions the activity of p53 is maintained at low levels by its negative regulator Mdm2. Mdm2 binds to p53 and mediates its ubiquitination which allows its recognition by 26S proteasomes where p53 is digested into oligopeptides.

Crystallographic analysis of the p53-Mdm2 interaction revealed the biochemical basis of the Mdm2-mediated inhibition of p53. Mdm2 can antagonize p53 by concealing its transactivation domain. It achieves this by binding to an N-terminal region of p53 thereby blocking its ability to associate with the transcriptional machinery (Oliner et al., 1993; Chi et al., 2005). Mdm2 shuttles between the nucleus and the cytoplasm (Roth et al., 1998). This shuttling activity required the nuclear export sequence of Mdm2 (Iwakuma and Lozano, 2003). Mutation of the nuclear export sequence of Mdm2 abolishes Mdm2 nuclear export and also p53 degradation, implying that Mdm2 binds p53 in the nucleus and transports it into the cytoplasm where it is degraded (Tao and Levine, 1999).

Mdm2 mediates monoubiquitination and polyubiquitination of p53 (Li et al., 2003). The level of Mdm2 determines which activity will be performed. Low levels of Mdm2 activity in unstressed cells induce nuclear export while high levels of Mdm2 induce p53 polyubiquitination and degradation in the nucleus in order to suppress p53 function (Brooks and Gu, 2006).

p300 which is considered to be an E4 ligase for p53 is required for polyubiquitination of p53. It associates with Mdm2 and finalizes the process of polyubiquitination (Grossman et al., 2003). PirH2, another E3 ubiquitin ligase, also antagonizes p53 function. It interacts with p53 and promotes p53 ubiquitination independent of Mdm2 (Leng et al., 2003). Deubiquitination of p53, on the other hand, is achieved by HAUSP (herpesvirus-associated ubiquitin-specific protease (Li et al., 2002).

### 1.3.2 Activation of p53

p53 is activated and stabilized in response to cellular stress. DNA damaging agents such as ionizing radiation, ultraviolet light or some anticancer drugs lead to rapid accumulation of the p53 protein (Giaccia and Kastan, 1998). Disruption of the interaction of p53 with its negative regulator Mdm2 is the first step in the p53 activation cascade. Ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) trigger a variety of post-translational modifications of p53 that promote its stabilization and activation. Phosphorylation of p53 at ser15 and ser20 after DNA damage, for instance, has been shown to inhibit the interaction between p53 and Mdm2 leading to p53 stabilization (Shieh et al., 1997; Chehab et al., 2000; Shieh et al., 2000; Schon et al., 2002). ATM furthermore phosphorylates the checkpoint kinase-2 (Chk2, Matsuoka et al., 2000) that is followed by phosphorylation of p53 at ser20 (Chehab et al., 1999; Hirao et al., 2000). Protein kinases like ATM and ATR furthermore activate p53 by mediating the degradation of Mdm2 (Stommel and Wahl, 2004).

Phosphorylation is indeed the most studied posttranslational modification of p53. Ser33, one of p53 phosphorylation sites, was found to be constitutively phosphorylated by glycogen synthase kinase-3 beta (GSK-3β) and by the cdk-activating kinase (CAK) complex (Ko et al., 1997; Turenne and Price, 2001). It was also reported that p53 is phosphorylated at thr55 by the TATA-box-binding protein-associated factor 1 (TAF1). Phosphorylation of this site enhances the association of p53 with CRM1 (chromosomal region maintenance-1), a protein that triggers cytoplasmic translocation of p53 (Gatti et al., 2000; Li et al., 2004; Cai and Liu, 2008). Phosphorylation of Mdm2 also has an impact on p53 activity. Phosphorylation of Mdm2 at ser395, for instance, reduces the translocation of p53 from the nucleus to the cytoplasm, resulting in increased p53 stability (Gajjar et al., 2012).

Acetylation is, furthermore, a critical post-translational modification for p53 activity that increases the sequence-specific binding of p53 to DNA (Sykes et al., 2006). The three acetyltransferases p300/CBP (cAMP-response element binding protein (CREB)-Binding protein), PCAF (p300/CBP-associated factor) and hMOF/TIP60 (human males absent on the first/Tat-interactive protein of 60 kDa) play a pivotal role in p53 activation (Scolnick et al., 1997; Lin et al., 1999; Legube et al., 2004). To induce transcription of its target genes, p53 needs to interact with co-factors of transcription. p300/CBP, p53 and PML form a complex in nuclear bodies that is required for p53 acetylation by p300/CBP (Pearson et al., 2000). This association of p300/CBP with p53 is enhanced by N-terminal phosphorylations (Polley et al., 2008; Jenkins et al., 2009). Lys164, lys305, lys370, lys372, lys373, lys381, lys382, and lys386 can all be acetylated by p300/CBP (Gu et al., 1997). This acetylation of p53 by p300/CBP induces its pro-apoptotic activity (Knights et al., 2006). In response to DNA damage, PTEN (phosphatase and tensin homolog) forms a complex with p300/CBP and this enhances p300/CBP activity and acetylation of p53 (Li et al., 2006). p300/CBP furthermore cooperates with Mdm2 and function as an E4 ubiquitin ligase to promote p53 polyubiquitination (Shi et al., 2009, Grossman et al., 2003). In contrast to p300/CBP, PCAF and hMOF/TIP60 do not need special conditions to function. According to the regulation of p53 by acetylation, p53 can

also be regulated by histone deacetylases (HDACs). Protein complexes containing HDAC1 or NAD-dependent protein deacetylase sirtuin-1 (SIRT1) strongly repress p53-dependent transcription and reverse p53 dependent growth arrest and apoptosis in response to DNA damage (Juan et al., 2000; Luo et al., 2000; Luo et al., 2001; Vaziri et al., 2001). Mdm2 can recruit HDAC1 to p53 and facilitate HDAC1-mediated deacetylation of p53 (Ito et al., 2002).

p53 is also modified by methylation, sumoylation and neddylation. p53 is, for instance, methylated at lys370 by Smyd2 (SET and MYND domain-containing protein 2), at lys372 by SET 7/9, and at lys382 by SET8 (Chuikov et al., 2004; Huang et al., 2006; Ivanov et al., 2007; Shi et al., 2007; Kurash et al., 2008). Methylation of lys370 and lys382 inhibits DNA binding and transcriptional activity of p53 and methylation of lys382 impairs acetylation of this site (Huang et al., 2006; Shi et al., 2007).

The activity of p53 depends on its subcellular localization and both, nuclear export and import of p53 are tightly regulated (O'Brate and Giannakakou, 2003). The shuttling of p53 between the nucleus and cytoplasm is regulated by the nuclear localization signal (NLS) and the nuclear export signal (NES, Liang and Clarke, 1999; Stommel et al., 1999; Zhang and Xiong, 2001). p53 is synthesized in the cytoplasm and needs to be transported into the nucleus to exert its transcriptional activity after cellular stress. This accumulation in the nucleus is important for its tumor suppressive activity. Cytoplasmic sequestration of p53 was observed in certain tumors like neuroblastomas and in breast and colon cancer (Moll et al., 1992; Bosari et al., 1995; Moll et al., 1995; Ostermeyer et al., 1996). After cellular stress, like DNA damage, p53 gets imported into the nucleus through its NLS, undergoes tetramerization and binds to and activates its target genes (Jimenez et al., 1999). The p53 tetramer masks its NES and thereby blocks nuclear export (Stommel et al., 1999). Several proteins can influence p53 subcellular localization. One of the most important of these proteins is Mdm2. Mdm2 also contributes to the cytoplasmic sequestration of p53 in some tumors (Lu et al., 2000).

Beside DNA damage, p53 is also induced and activated in response to activated oncogenes. p19ARF, the alternate reading frame product of the p16/INK4A locus is a major mediator of this induction. p19ARF interacts with Mdm2 upon oncogenic stress and sequesters it in the nucleolus and thus away from p53 (Weber et al., 1999; Sherr, 2006). Since loss of p19ARF does not prevent the activation of p53 in response to DNA damage, it is most likely that oncogenic stress and DNA damage use different and independent pathways for activating p53 (Stott et al., 1998; Kamijo et al., 1999).

## 1.4 The physiologic functions of p53

The p53 tumor suppressor protein is involved in many cellular processes including cell differentiation, cell cycle regulation, apoptosis, senescence, DNA repair and metabolism (Shaw, 1996; Fridman and Lowe, 2003; Meek, 2004; Teodoro et al., 2007; Vousden and Ryan, 2009; Rufini et al., 2013). These activities are mediated by different activities of p53 such as transcriptional activation, transrepression and exonuclease activity. A large set of p53 target genes is involved in these cellular processes (Figure 1.6, Amundson et al., 1998; Giacca and Kastan, 1998). The high p53 levels induced by various stress types prevent inappropriate proliferation of cells carrying damaged DNA. Transcription-dependent and transcription- independent mechanisms in the nucleus and mitochondria contribute to the prevention of cell proliferation in response to stress (Vousden, 2002; Mihara et al., 2003).

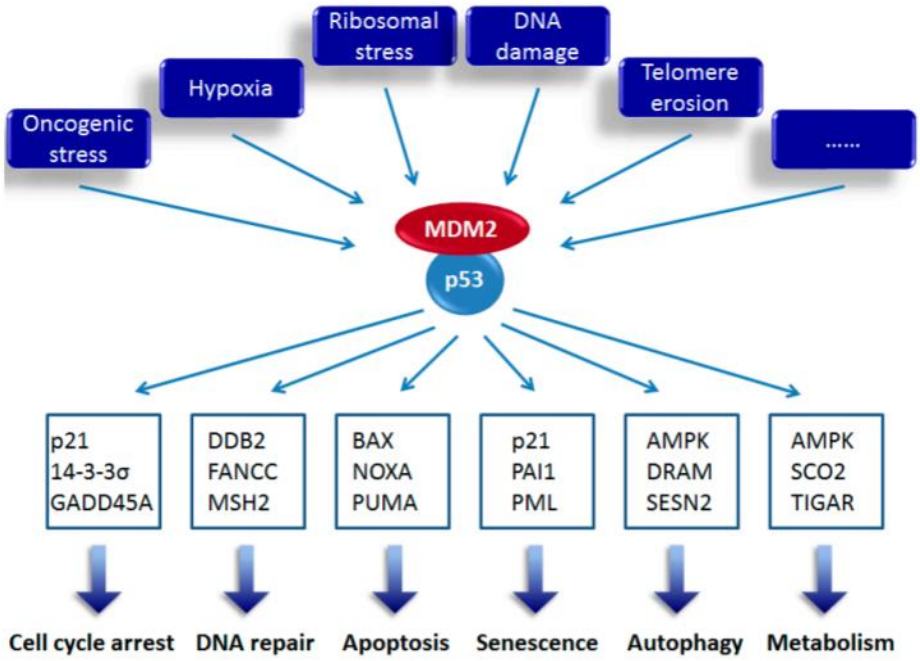


**Figure 1.6: Activation of p53 and induction of p53 target genes.** In response to various stress signals, such as oncogenic stress, hypoxia, ribosomal stress, DNA damage, telomere erosion and others, p53 becomes activated and induces the expression of an array of target genes including *p21*, *PUMA*, *DRAM*, *DDB2* and *AMPK*. The activation of p53 target genes leads to biological responses such as cell cycle arrest, DNA repair, apoptosis, senescence, autophagy or alteration of cell metabolism (From: Hao and Cho, 2014).

### 1.4.1 Transcription-dependent activities of p53

#### *1.4.1.1 p53-dependent induction of cell cycle arrest*

The cell cycle is regulated at two major check points: a checkpoint at the G1/S boundary, after which cells can proceed into S-phase and become committed to DNA synthesis, and a checkpoint at the G2/M boundary, after which cells progress to mitosis (Barnum and O'Connell, 2014). The critical signals for both transitions result from the cell cycle-specific phosphorylation of several substrates including pRb in the case of the G1/S boundary and tyrosine kinases and histone H1 in the case of the G2/M transition (Nigg, 1993a). These phosphorylations are mediated by p34cdc2 protein kinases (Nigg, 1993b). These cell cycle regulators were initially identified as the products of the two cell division cycle (cdc) genes *cdc2* from the

fission yeast *Saccharomyces pompe* and *cdc28* from the budding yeast *Saccharomyces cerevisiae* (Mendenhall and Hodge, 1998). The activity of these kinases requires their association with cyclins that act as essential activation subunits (Reed et al., 1992).

The cyclin family is divided into three classes, based on their physiological function. D-type cyclins are required during the G1 phase of the cell cycle. A-type cyclins guide the progression through the S-phase and B-type cyclins are active during mitosis (Malumbres, 2014). In contrast to yeast cells, where a single p34cdc2/cdc28 kinase controls both the G1/S and the G2/M transition, vertebrates possess a multi-gene family of homologous kinases that control the cell cycle (Meyerson et al., 1992). Cell cycle progression is controlled by the sequential formation, activation and inactivation of cyclin-cdk complexes (Vermeulen et al., 2003). The discovery of a family of cdk inhibitors suggested a mechanism by which the activity of cyclin-cdk complexes can be controlled. Such inhibitors bind to cyclin- cdk complexes and inhibit their kinase activity (Hunter, 1993). The first of the cdk-inhibitors that has been discovered and characterized was p21/WAF1/CIP1/Sdit (wild-type p53 activated fragement-1/cyclin-dependent kinase (cdk)-interacting protein-1), a p53 regulated gene (Harper et al., 1993). p21-deficient cells show an aberrant G1 arrest after exposure to radiation (Deng et al., 1995). p21 also contributes to the G2 arrest by inhibiting the cyclinB1/cdc2 activity that is crucial for cells to enter mitosis (Dash et al., 2005). p21 is up-regulated by p53 in response to DNA damage (DiLeonardo et al., 1994) and is considered to play an important role in the p53-mediated growth arrest (El-Deiry et al., 1994). Loss of the G1/S checkpoint or impaired G2 arrest as a result of p53 inactivation can give rise to aneuploid or polyploid cell indicating that the ability of p53 to halt cells at different checkpoints of the cell cycle is vital for its tumor suppressive activity (Bunz et al., 1998; Meek, 2000). p21 is furthermore necessary for p53-mediated repression of genes including *cdc2*, *cdc25C* and *tert* (telomerase reverse transcriptase, Löhr et al., 2003; Shats et al., 2004). Regulation of p21 by p53 apparently occurs at the level of transcription as the ability of p53 to induce growth arrest requires its transactivation domain (Pietenpol et al., 1994). Loss of p53 results in a dramatic reduction in p21 levels (El-Deiry et al., 1994).

*1.4.1.2 p53-dependent induction of apoptosis*

Apoptosis, also called "programmed cell death" is a special form of cell death (Kerr, 2002). Several target genes of p53 including *bcl-2* (B-cell lymphoma 2), *bax* (bcl-2-associated X protein), *puma* (p53-upregulated modulator of apoptosis), *pmaip1* (phorbol-12-myristate-13-acetate-induced protein 1) also known as *noxa* and *bid* (BH3-interacting-domain) contribute to the induction of p53-dependent apoptosis (Miyashita et al., 1994; Oda et al., 2000; Nakano and Vousden, 2001; Say et al., 2002). The *bax* gene promoter responds to p53 activation because of the presence of a p53 binding site (Miyashita et al., 1994). Thereafter accumulates the pro-apoptotic Bax protein in mitochondria. The activated Bax protein assembles a protein complex that is called the permeability transition pore (PTP). This creates a channel through both membranes of the mitochondria resulting in swelling of the mitochondrial matrix and eventually in rupture of the mitochondria outer membrane (Schwarz et al., 2007). Ionizing radiation also induces *noxa* in a p53-dependent manner. The Noxa protein contains a BH3 (Bcl-2-homology domain 3) motif that is found widely on Bcl-2 family members. Noxa localizes to mitochondria and interacts with anti-apoptotic Bcl-2 family members like Bcl-XL (B-cell lymphoma- extra-large) and Mcl-1 (myeloid leukemia cell differentiation protein) through its BH3 domain. Puma also encodes a BH3-containing protein that localizes to mitochondria. With its BH3 domain Puma interacts with Bcl-2 and Bcl-XL and induces apoptosis by inducing Cytochrome C release and activation of Caspase 3 and 9 (Benchimol, 2001).

*1.4.1.3 p53-dependent regulation of cellular metabolism*

Fatty acids are important metabolic intermediates that play a pivotal role in lipid synthesis. Fatty acids are, moreover, used in the mitochondria to generate ATP through beta oxidation. In response to genotoxic stress and glucose deprivation, p53 modulates the oxidation of fatty acids by increasing guanidinacetate N-methyltransferase levels. Guanidinacetate N-methyltransferase in turn converts guanidinacetate to creatine and this enhances the energy metabolism (Zhu and Prives, 2009).

### 1.4.2 Transcription-independent activities of p53

Beside transcription-dependent cellular responses, p53 can also induce apoptosis in the cytoplasm (Arima et al., 2005; Speidel et al., 2006). Particularly after DNA damage, p53 shuttles to mitochondria where it associates with members of the Bcl-2 family (Mihara et al., 2003; Chipuk et al., 2004). This association of p53 with members of the Bcl-2 family leads to the permeabilization of the outer membrane of mitochondria and to the release of Cytochrome C (Wolff et al., 2008).

## 1.5 Mutant p53

p53 is mutated in about 50% of all human cancers (Rivlin et al., 2011). In contrast to other tumor suppressor genes that are usually inactivated in cancer, the majority of cancer-associated mutations of the *p53* gene are missense mutations (Weinberg, 1991; Harris and Hollstein, 1993). Most of these missense mutations are clustered within the central DNA binding domain (Cho et al., 1994). Wintin the DNA binding domain there are several mutation hot spots including R175, G245, R248, R249, R273 and R282 (Olivier et al., 2002).

In 1993, it was observed that mutant p53 shows a gain of function when mutant p53 was transfected into p53 null cells (Dittmer et al., 1993). This result that mutant p53 is not silent but has frequently gained novel, tumorigenic activities was further supported by the observation that patients carrying a p53 missense mutation in the germline have a significantly earlier onset of cancer than patients with mutations in the p53 gene that result in loss of p53 protein expression (Bougeard et al., 2008; Zerdoumi et al., 2013). Likewise, mice that expressed mutant p53 showed a tumor profile that was more metastatic and aggressive than mice that expressed wild-type p53 or mice with two deleted alleles of *p53* (Lang et al., 2004; Olive et al., 2004; Doyle et al., 2010; Morton et al., 2010). Xenografts harboring mutant p53, furthermore, also showed enhanced tumor invasion (Adorno et al., 2009; Muller et al., 2009).

How mutant p53 exert its pro-oncogenic effect is not fully understood. The first model that has been proposed says that mutant p53 retains a residual transactivation activity and has an impact on chromatin by inducing MLL1/2 (mixed lineage leukemia protein 1/2) and MOZ (monocytic leukemia zinc finger protein (Zhu et al., 2015). The second model that has been proposed is that certain unstructured mutants of p53 sequester other proteins that enable mutant p53 to bind p63 or p73 resulting in changes in the transcriptional profiles that e.g. alter receptor tyrosine kinase signaling resulting in invasion and metastasis (Muller et al., 2009; Weismueller et al., 2014). The third model proposes that mutant p53 can cooperate with the SWI/SNF complex (Switch/Sucrose non-fermentable) to upregulated VEGFR2 (vascular endothelial growth factor) that is considered as an important regulator of angiogenesis (Pfister et al., 2015).

## 1.6 Family with sequence similarity 83

The Fam83 (Family with sequence similarity 83) family is a family of proteins that includes eight members (Fam83A-H). The Fam83 family is characterized by the presence of a highly conserved domain, the DUF 1669 (domain of unknown function) domain (Cipriano et al., 2014). Each member of the Fam83 family is located at a distinct genomic site. *fam83* genes are only found in jawed vertebrates. There are no homologues or orthologues of *fam83* in primitive organisms such as *Saccharomyces cerevisiae* or *Drosophila melanogaster* (Bartel et al., 2016). Several members of the Fam83 protein family have oncogenic properties (Cipriano et al., 2012; Grant, 2012; Cipriano et al., 2013; Cipriano et al., 2014; Bartel et al., 2016; Snijders et al., 2017). Fam83A, for example, is interacting with c-Raf and PI3Kp85 (Phosphoinosite-3-kinase) and its overexpression in cancer is correlated with poor prognosis of breast cancer patients (Lee et al., 2012; Bartel and Jackson, 2017). By activating TGF-β and Wnt signaling, Fam83A plays a vital role in pancreatic cancer (Chen et al., 2017). Fam83B, Fam83C and Fam83E were identified as potential oncogenes that transform immortalized mammary epithelial cells (Snijders et al., 2017). Fam83B has also been shown to activate MAPK (mitogen-activated protein

kinase) signaling (Cipriano et al., 2014). Many human tumors show high levels of Fam83B mRNA. Ablation of Fam83B inhibited proliferation, anchorage-independent growth and tumorigenicity of breast cancer cells (Cipriano et al., 2012). Fam83D (also called C20orf129 or CHICA) localizes to spindle microtubules and plays a role in the distribution of sister chromatids (Sauer et al., 2005). Fam83D-deficient cells show malfunctioning of the spindle resulting in enhanced chromosome missegregation and aneuploidy (Santamaria et al., 2008). Fam83D is amplified and overexpressed in many human cancers (Liao et al., 2015; Walian et al., 2016). Forced expression of Fam83D in non-malignant cells reduces the expression of the *fbxw7* (F-box and WD repeat domain-containing protein 7) tumor suppressor gene and promotes proliferation and invasion (Wang et al., 2013). Fam83H is a keratin-associated protein that regulates the organization of the cytoskeleton. Cancer cells with overexpression of Fam83H loose cell polarity and keratin filaments and show increased invasion and metastasis of colorectal cancer (Kuga et al., 2013).

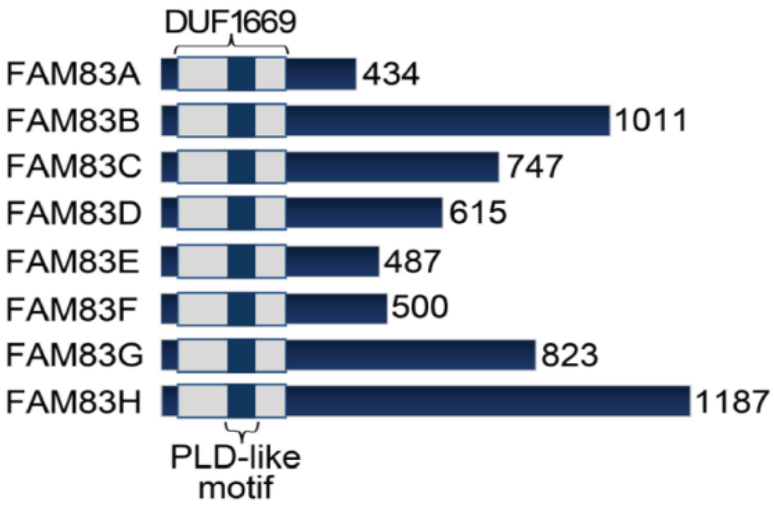


**Figure 1.7: The Fam83 protein family.** Members of the Fam83 protein family are characterized by the highly conserved N-terminal domain of unknown function 1669 (DUF1669, from: Bartel et al., 2016).

Analysis of the human proteome indicates that members of the Fam83 protein family are expressed in several tissues, yet at low levels, particularly in comparison with other proto-oncogenes such as the epidermal growth factor receptor (EGFR) or K-Ras (Kirsten rat sarcoma; Bartel et al., 2016).


**Figure 1.8: Expression of Fam83 family members.** The table shows expression of the Fam83 family members Fam83A-H and of the epidermal growth factor receptor (EGFR), K-Ras (Kirsten rat sarcoma) and beta actin. The tissue types include the blood and immune system, the nervous system, the muscoskeletal system, the internal and secretory system and the reproductive system (from: Bartel et al., 2016).

### 1.6.1 Family with sequence similarity 83 member F

The *fam83F* gene is found on chromosome 22q13.1. Forced expression of *fam83F* in H1299 cells was observed to increase p53 abundance (Zhang et al., 2015). More recently, it was found that *fam83F* expression was increased in esophageal squamous cell carcinoma. Introduction of miR-143 into esophageal cancer cells down-regulated *fam83F* expression which resulted in inhibition of cell proliferation, migration and invasion (Mao et al., 2016). miR-143 is downregulated and functions as a tumor suppressor in several tumors including bladder cancer, cervical cancer

and prostate cancer (Noguchi et al., 2011; Chen et al., 2014; Zhou et al., 2015). The 3'UTR of Fam83F mRNA is a direct target of miR-143. Overexpressing or silencing miR-143 in esophageal squamous cell carcinoma cells showed that miR-143 regulates Fam83F expression by decreasing the stability of its mRNA (Mao et al., 2016). Fam83F is upregulated in head-and neck cancer, breast and lung cancer and increased expression of Fam83F is associated with poor patient survival (Snijders et al., 2016).

## 1.7 Aim of the project

p53 is regulated by a complex network of positive and negative regulators and the mechanism by which p53 is controlled in different cell types and growth conditions is not fully understood. Screening a cDNA library from the teleost Medaka for novel regulators of p53 showed for the first time that Fam83F is a novel regulator of p53 (Zhang et al., 2015).

The aim of this study is to investigate the molecular mechanism by which Fam83F regulates p53 abundance and to explore its function in the context of p53.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals and consumables

| Chemicals | Source |
|---|---|
| Acetic Acid | Merck, Darmstadt |
| Acetone | Roth, Karlsruhe |
| Adenosine 5′-triphosphate (ATP) disodium Salt | Sigma-Aldrich, Taufkirchen |
| Agar | Nordwald, Erlangen |
| Agarose | Peqlab, Erlangen |
| Amersham ECL Hyperfilm | GE Healthcare, Freiburg |
| Amersham™ ECL™ Prime Western Blotting Detection Reagent (Highly chemiluminescent detection reagent) | GE Healthcare Life Sciences,Freiburg, Germany |
| Ammonium persulfate (APS) | Sigma-Aldrich, Taufkirchen |
| Ampicillin, sodium salt | Roth, Karlsruhe |
| Aqua-Poly/Mount | Polysciences Europe GmbH, Germany |
| β-mercaptoethanol | Roth, Karlsruhe |
| Blotting Filter Paper | Bio-Rad, München |
| Bovine serum albumin (BSA) | PAA Laboratories GmbH, Pasching |

| | |
|---|---|
| Bovine Donor Serum | Gibco, Invitrogen,Karlsruhe |
| Bromophenol blue | Sigma-Aldrich, Taufkirchen |
| Cell culture plastic ware (flasks and dishes) | Greiner Bio-One, Frickenhausen |
| Coelenterazine | Sigma-Aldrich, Taufkirchen |
| p-Coumaric Acid | Sigma-Aldrich, Taufkirchen |
| Crystal Violet (certified by the Biological Stain Commission) | Sigma-Aldrich, Taufkirchen |
| Cycloheximide | Serva, Heidelberg |
| Dimethylsulfoxide (DMSO) | Roth, Karlsruhe |
| Dithiothreitol (DTT) | Roth, Karlsruhe |
| 6×DNA loading dye | PeqLab, Erlangen |
| dNTP | Mix Long Range Peqlab,Erlangen |
| Draq5 | BioStatus Limited, Shepshed (UK) |
| Dulbecco's Modified Eagle Medium (DMEM) | Invitrogen, Karlsruhe |
| Ethanol | Roth, Karlsruhe |
| Ethidium Bromide | Roth, Karlsruhe |
| Etoposide | Sigma-Aldrich, Taufkirchen |
| EthylenediamineTetraacetic Acid (EDTA) | Roth, Karlsruhe |

| | |
|---|---|
| Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetra acetic acid (EGTA) | Roth, Karlsruhe |
| Foetal Bovine Serum (FBS) | PAA, Pasching, Austria |
| Formaldehyde | Merck, Darmstadt |
| G418, Geneticin® | Thermo Fisher Scientific, (Karlsruhe) GmbH. |
| Glucose | Roth, Karlsruhe |
| Glutathione sepharose 4B | GE Healthcare, Dharmacon |
| L-Glutathione | Roth, Karlsruhe |
| Glycerol | Roth, Karlsruhe |
| Glycyl-glycine (Gly-Gly) | Sigma-Aldrich, Taufkirchen |
| Guanidinium-HCL | Sigma Aldrich, Taufkirchen |
| HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) | Roth, Karlsruhe |
| Hydrogen Chloride (HCl) | Roth, Karlsruhe |
| Hydrogen Peroxide ($H_2O_2$) | Roth, Karlsruhe |
| Hydroxyurea | Sigma Aldrich, Taufkirchen |
| Igepal CA-630 (Nonident P-40 (NP40)) | Roth, Karlsruhe |
| Imidazole | Sigma Aldrich, Taufkirchen |
| Immun-Blot® PVDF | Bio-Rad, München |
| ImmunoPureR Immobilized Protein A | Pierce, Thermo Scientific, Rockford (USA) |
| ImmunoPureR Immobilized Protein G | Pierce, Thermo Scientific, Rockford (USA) |

| | |
|---|---|
| Iodoacetamide | Sigma Aldrich, Taufkirchen |
| Ink | Pelikan, Hannover |
| Isopropanol | Roth, Karlsruhe |
| Luminol sodium salt | Roth, Karlsruhe |
| Luciferase Substrate | Promega, Mannheim |
| Lysozyme | Roth, Karlsruhe |
| Magnesium carbonate ($MgCO_3$) | Roth, Karlsruhe |
| Magnesium Chloride ($MgCl_2$) | Roth, Karlsruhe |
| Magnesium Sulfate ($MgSO_4$) | Sigma Aldrich, Taufkirchen |
| Methanol | Roth, Karlsruhe |
| Methyl methanesulfonate (MMS) | Fluka Chemical GmbH, Switzerland |
| β-mercaptoethanol | Roth, Karlsruhe |
| MG132 | Calbiochem, Darmstadt, Germany |
| N,N,N',N'Tetramethylethylenediamine (TEMED) | Roth, Karlsruhe |
| $Ni^{2+}$nitrilotriacetic acid (Ni-NTA)-agarose | Qiagen Hilden, Germany |
| Parafilm M | Brand, Wertheim |
| Passive Lysis Buffer | Promega, Mannheim |
| PCR plate and PCR clear seal | Steinbrenner, Wiesenbach |
| Penicillin/Streptomycin | Invitrogen, Karlsruhe |

| | |
|---|---|
| Petri dishes | Greiner Bio-One, Frickenhausen, Germany |
| 1,10-Phenanthroline | Sigma Aldrich, Taufkirchen |
| Phenylmethanesulfonyl fluoride (PMSF) | Sigma Aldrich, Taufkirchen |
| Phosphostop Phosphatase Inhibitor Cocktail | Roche, Grenzach |
| Phosphate buffered saline (PBS) | Invitrogen, Karlsruhe |
| Pipette tips | Brand, Wertheim |
| Potassium Chloride (KCl) | Roth, Karlsruhe |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Roth, Karlsruhe |
| Puromycin | Sigma Aldrich, Taufkirchen |
| QuantiTect Green PCR SYBR | Qiagen, Hilden, Germany |
| Reaction tubes (1.5 ml and 2 ml) | Eppendorf, Hamburg |
| Reaction tubes (15 ml and 50 ml) | Greiner Bio-One, Frickenhausen |
| Rotiphorese Gel 30: Acrylmide/bis-Acrylamide | Roth, Karlsruhe |
| Roti®-Free Stripping Buffer 2.2 plus | Roth, Karlsruhe |
| Roti®-Quant(Protein quantitation assay according to Bradford) | Roth, Karlsruhe |
| RNase Inhibitor (rRNasin) | Promega, Mannheim |
| ScreenFect® | ScreenFect GmbH, Karlsruhe |
| Skimmed milk powder | Saliter, Obergünzburg |
| Sodium acetate (NaAc) | Roth, Karlsruhe |

| | |
|---|---|
| Sodium bicarbonate (NaHCO3) | Roth, Karlsruhe |
| Sodium chloride (NaCl) | Roth, Karlsruhe |
| Sodium deoxycholate. | Roth, Karlsruhe |
| Sodium dihydrogen phosphate($NaH_2PO4$) | Roth, Karlsruhe |
| Sodium dodecyl sulfate (SDS) | Roth, Karlsruhe |
| Sodium hydrogen phosphate ($Na_2HPO4$) | Roth, Karlsruhe |
| Sodium Hydroxide (NaOH) | Roth, Karlsruhe |
| Sucrose | Roth, Karlsruhe |
| Syringes and needles | B. Braun, Melsungen |
| Thio-urea | Roth, Karlsruhe |
| Trichloroacetic acid (TCA) | Sigma-Aldrich, Taufkirchen |
| Tris-base | Roth, Karlsruhe |
| Triton X-100 | Roth, Karlsruhe |
| Tryptone/Peptone | Roth, Karlsruhe |
| Tween 20 | Sigma Aldrich, Taufkirchen |
| Urea | Roth, Karlsruhe |
| White flat-bottomed96-well plate | Greiner, Tecan, Salzburg, Austaria |

### 2.1.2 Enzymes

| Enzyme | Source |
|---|---|
| DNase I | Promega, Mannheim |
| M-MLV Reverse Transcriptase, RNase H(-) | Promega, Mannheim |
| Restriction enzymes: BamHI, NotI and XhoI | Fermentas, St Leon-Rot, Germany |
| RNase-A | Invitrogen, Karlsruhe |
| T4 DNA Ligase | Promega, Mannheim |
| 0.25% Trypsin-EDTA | Invitrogen, Karlsruhe |
| GoTaq® G2 DNA Polymerase | Promega, Mannheim |
| PrimeStar DNA polymerase | Promega, Mannheim |

### 2.1.3 Kits

| KIT | Source |
|---|---|
| QIAGEN Plasmid Maxi kit(plasmid purification) | Qiagen, Hilden, Germany |
| QIAprep Spin Miniprep Kit (plasmid purification) | Qiagen, Hilden, Germany |
| QIAquick Gel Extraction Kit | Qiagen, Hilden, Germany |
| RNeasy Mini Kit | Qiagen, Hilden, Germany |

### 2.1.4 Oligonucleotides

*2.1.4.1 Primers*

All primers were synthesized by Metabion (Martinsried, Germany).

2.1.4.1.1 Primers for cloning

| Primer | Sequence |
|---|---|
| Fam83F-NotI-F | 5′-CTT GCG GCC GCG ATG GCC GAG TCC CAG CTG AAC-3′ |
| Fam83F-Flag-BamHI-R | 5′-CGG GAT CCT CAG GAA ATC ACA CAG TTG CTA G-3′ |
| Fam83F-BamHI-F | 5′-CTT GGA TCC GAT GGC CGA GTC CCA GCT GAA C-3′ |
| Fam83F-Flag-R-Δ1 | 5′-CTT GCG GCC GCT TAT TTA TCA TCA TCA TCT TTA TAA TCA AAG GGC TCT ACG TTC TG-3′ |
| Fam83F-Flag-R-Δ2 | 5′-CTT GCG GCC GCT TAT TTA TCA TCA TCA TCT TTA TCC TCT CCC AAG GGG ATG GGC T-3′ |
| GST-Fam83F-BamHI-F | 5′-CCG CGT GGA TCC CCA ATG GCC GAG TCC CAG CTG AAC TGC-3′ |
| GST-Fam83F-XhoI-R | 5′-CCG CTC GAG TCA GGA AAT CAC ACA GTT GCT AG-3′ |

2.1.4.1.2 Primers for qRT-PCR

| Primer | Sequence |
|---|---|
| RT-β-Actin- Forward | 5′- CCAACCGCGAGAAGATGA-3′ |
| RT-β-Actin- Reverse | 5′- CCAGAGGCGTACAGGGATAG-3′ |
| RT-Mdm2- Forward | 5′- TGGAGTCCCGAGTTTCTCTG-3′ |
| RT- Mdm2- Reverse | 5′- AGCCACTAAATTTCTGTAGATCATTG-3′ |
| RT-P53- Forward | 5′- CCCCAGCCAAAGAAGAAAC-3′ |
| RT- P53- Reverse | 5′- AACATCTCGAAGCGCTCAC-3′ |
| RT-Fam83F-Forward | 5′-CGTCGGCTTCTACATGCCC-3′ |
| RT-Fam83F-Reverse | 5′-GCTCTACGTTCTGTCCTGTCA-3′ |

*2.1.4.2 Lentiviruses carrying shRNAs*

All lentiviruses were produced by Sigma Aldrich, Saint Louis Missouri, USA.

| Name | TRC number | Clone ID | Sequence |
|---|---|---|---|
| **Control shRNA** | SHCO16V-1EA | | MISSION® pLKO.1-puro non -target shRNA control transduction particles targets no known genes from any species |
| **Fam83F shRNA** | TRCN-0000434615 | NM_138435.2-1667s21c1 | 5′-CCG GGT CGC ACA CTG CAC CAG TTT GCT CGA GCA AAC TGG TGC AGT GTG CGA CTT TTT TG-3′ |

### 2.1.5 Bacteria

| Name | Genotype |
|---|---|
| BL21 (DE3) | *E. coli* $F^-$ompT gal $hsdS_B$ ($r_B^-m_B^+$) λ (DE3[lacI lacUV5- T7 gene l indl sam7 nin5]) |
| DH5α | *E. coli* F- and A1 glnV44thi-1 recA1 relA1 gyrA96 deoRnupG Φ80dlacZΔM15 Δ(lacZYA-argF) U169, $hsdR17(r_k^-m_k^+)$, λ |

### 2.1.6 Eukaryotic cells and cell lines

| Cell line | Origin | P53 status | Culture medium |
|---|---|---|---|
| GM38 | Human normal skin fibroblast | wild type | DMEM + 15% FCS |
| H1299 | Human non-small cell lung Carcinoma | Null | DMEM + 10% FCS |
| MCF7 | Human breast adenocarcinoma cell line | wild type | DMEM + 10% FCS |
| RKO | Human colon carcinoma | wild type | DMEM + 10% FCS |

### 2.1.7 Plasmids

| Name | Description |
|---|---|
| pBluescriptR-FAM83F | Vector containing the human FAM83F coding sequence (Thermo Scientific) |
| pcDNA3 | Mammalian Expression Vector (Invitrogen) |
| P3xFLAG-CMV™ 7.1 | Mammalian Expression Vector (Invitrogen) |

| | |
|---|---|
| His-Ubiquitin | Expression plasmid containing the cDNA of His-Ubiquitin carrying a 6x His-tag, (provided by Sibylle Mittnacht) |
| pcDNA3-Mdm2 | Expression plasmid containing the cDNA of murine Mdm2 (provided by Arnold Levine) |
| pcDNA3-p53 | Expression plasmid containing the cDNA of P53 |
| pcDNA3-p53-R273H | Expression plasmid containing the cDNA of mutant P53 where the arginine 273 was replaced by histidine |
| pcDNA3-p53-R175H | Expression plasmid containing the cDNA of mutant P53 where the arginine 175 was replaced by histidine |
| Flag Fam83F | Expression plasmid containing the cDNA of FLAG- tagged human FAM83F |
| Flag Fam83F Δ-1 | Expression plasmid containing the cDNA of FLAG- tagged human FAM83F deletion mutant (aa 1-397) |
| Flag Fam83F Δ-2 | Expression plasmid containing the cDNA of FLAG- tagged human FAM83F deletion mutant (aa 1-265) |
| V5-p53 | Expression plasmid containing the cDNA ofV5 tagged human p53 |
| V5-p53Δ-1 | Expression plasmid containing the cDNA ofV5 tagged human p53 deletion mutant (aa 1-300) |
| V5-p53Δ-2 | Expression plasmid containing the cDNA ofV5 tagged human p53 deletion mutant (aa 1-160) |

## 2.1.8 Antibodies

*2.1.8.1 Primary antibodies*

| Target | Description | Source |
|---|---|---|
| Bax | Rabbit, polyclonal | Cell Signalling, Danvers, USA |
| Fam83F (K18) | Rabbit, polyclonal | Santa Cruz, Heidelberg |
| Flag (F3165) | Mouse, monoclonal | Sigma-Aldrich, Taufkirchen |
| Flag (DYKDDDDK) Affinity gel | Mouse, monoclonal | Biotool (Biotool.com) |
| GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (6C5) | Mouse, Monoclonal | HyTest, Turku, Finland |
| HA | Rat, Monoclonal | Roche Diagnostics, USA |
| Histone-H3 (C16)(sc-8654) | Goat, Polyclonal | Santa Cruz, Heidelberg |
| MDM2 (4B2) | Mouse, Monoclonal | Calbiochem, Merck, Darmstadt |
| P21 | Mouse, Monoclonal | BD Pharmigen $^{TM}$ |
| P53(Ab-2) | Mouse, Monoclonal | Calbiochem, Merck, Darmstadt |
| P53 (DO-1) | Mouse, Monoclonal | Santa Cruz, Heidelberg |
| PCNA (PC10) | Mouse, Monoclonal | Santa Cruz, Heidelberg |

| | | |
|---|---|---|
| V5 | Mouse, Monoclonal | Serotec |
| Normal mouse IgG (control IgG) | Mouse, polyclonal | Santa Cruz, Heidelberg |
| Normal rabbit IgG (control IgG) | Rabbit, polyclonal | Santa Cruz, Heidelberg |

*2.1.8.2 Secondary antibodies*

| Secondary antibody | Source |
|---|---|
| Alexa Fluor 488 goat anti-mouse IgG | Invitrogen, Karlsruhe |
| Alexa Fluor 546 goat anti-rabbit IgG | Invitrogen, Karlsruhe |
| Clean-Blot™ IP Detection Reagent (HRP) | ThermoFisher Scientific (Karlsruhe) GmbH |
| Rabbit anti-goat IgG/HRP | Dako, Glostrup (Dennmark) |
| Goat anti-mouse IgG/HRP | Dako, Glostrup (Dennmark) |
| Goat anti-rabbit IgG/HRP | Dako, Glostrup (Dennmark) |

### 2.1.9 Size standards

| Name | Source |
|---|---|
| Peq-GOLD Protein Marker IV | Peqlab, Erlangen |
| Peq-GOLD 100bp DNA-Ladder-Mix | Peqlab, Erlangen |

### 2.1.10 Instruments and glassware

| Name | Source |
|---|---|
| Analytical balance | Mettler Toledo, Gießen |
| Bioruptor ® sonication device | Diagenode, Liege (Belgium) |
| Cell culture centrifuge for 15/50 ml Falcons | Universal 320, Centrifuge, Hettich |
| Cell culture incubator | Heraeus, Fellbach |
| ChemiDoc, Touch, imaging system | Bio-Rad, Laboratories GmbH, München |
| Clean bench | W. H. Mahl, Trendelburg |
| Cold room (4 ºC) | Foster, Schutterwald |
| Cold room (-20 ºC) | Foster, Schutterwald |
| Cooling centrifuge Biofuge Primo R for 15/50 ml falcons | Heraeus, Fellbach |
| Cooling centrifuge Eppendorf 5417 R for PCR tube | Eppendorf, Hamburg |
| Cooling microcentrifuge Heraeus Fresco17 | Thermo Scientific, Waltham (USA) |
| Corex® Centrifuge Tubes | Corex (USA) |
| Covaris™ S220 focused ultrasonicator | Thermo Scientific, Waltham (USA) |
| Electrophoresis equipment (SDS-PAGE) | Bio-Rad, München |
| Electrophoresis Power Supply | PeqLab, Erlangen |

| | |
|---|---|
| ELx 808IU Ultra Microplate (ELISA) Reader | Bio-Tek Instruments, Bad Friedrichshall |
| End-over-end rotator | Heidolph, Schwabach |
| EttanIPGphor II Isoelectric Focusing System | GE Healthcare, Freiberg |
| Floor centrifuge Beckman Avanti J-20 | Beckman Coulter, Krefeld |
| Freezer (-20 $^{o}$C) | Liebherr, Ochsenhausen |
| Freezer (-80 $^{o}$C) | New Brunswick Scientific, Edison (USA) |
| Glass pipettes | Brand, Wertheim |
| Glassware (Erlenmeyer flasks, beakers, bottles) | Scott, Mainz |
| Hamilton syringe | Hamilton, Martinsried |
| High speed floor centrifuge Avanti J2-HS | Beckman Coulter, Krefeld |
| Horizontal nucleic acid electrophoresis System | Bio-Rad, München |
| Inverted microscope | Leica, Wetzlar |
| KODAK X-OMAT 2000 X-ray film Processor | Kodak, Stuttgart |
| Laboratory balance | Sartorius, Göttingen |
| Luminometer plate reader | Perkin Elmer 1420 Luminescence Counter Victor 3v, Finland |
| Magnetic stirrer | IKA Labortechnik, Stauffen |
| Measuring cylinders (10 ml, 25 ml, 50 ml,100 ml, 250 ml, 500 ml, 1000 ml, 2000ml) | Brand, Wertheim |

| | |
|---|---|
| MicrocentrifugeHeraeus Pico 21 | Thermo Scientific, Waltham (USA) |
| NanoDrop ND-1000 Spectrophotometer | Marienfeld, Lauda Königshofen |
| Neubauer counting chamber | Marienfeld, Lauda-Königshofen |
| PCR thermocycler | Bio-Rad, München |
| Pipettes (2-20µl, 20-200µl, 200-1000µl) | Gilson, Middleton (USA) |
| Pipette Aid | Brand, Wertheim |
| Platform shaker | Heidolph, Schwabach |
| Real-time PCR System StepOnePlus | Applied Biosystems, Darmstadt |
| Refrigerator ($4^{o}$C) | Liebherr, Ochsenhausen |
| Shaker for Eppendorf tubes | Eppendorf, Hamburg |
| Test-tube rotator | Kisker, Steinfurt |
| Thermo-mixer | Eppendorf, Hamburg |
| Trans-Blot® Turbo ™ Transfer System | Bio-Rad, München |
| UV transilluminator | PeqLab, Erlangen |
| Vortex mixer | Julabo, Seelbach |
| Water bath-19L | Köttermann GmbH & Co KG |

### 2.1.11 Software

| Name | Version/Description |
|---|---|
| Image-J | version 1.45s |
| Image-lab | version 5.2.1 |
| KC4™ Data Analysis | version 3.01 |
| Zeiss LSM Image Browser | version 4.2.0.121 |

### 2.1.12 Data Bases

| Name | Source |
|---|---|
| Human Genome Assemblys | NCBI |

## 2.2 Methods

### 2.2.1 Cell culture and treatment

*2.2.1.1 Culture of human cells*

The cell lines and their respective culture media are listed in table 2.1.6. The cell culture medium was supplemented with fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured in sterile Cellstar® cell culture dishes at 37°C and 5% $CO_2$ in a humidified atmosphere. For sub-culturing, cells were detached from the culture dish by trypsinization. Therefore, the culture medium was removed from the cells. The cells were then washed with PBS. Trypsin/EDTA was added and incubated until the cells were detached. Trypsinization was stopped by the addition of complete growth medium. Then the cells were pelleted for 2 min at 1200 rpm. The cells were suspended in complete growth medium and a part of the cells was transferred to a new culture dish.

*2.2.1.2 Treatment of cells*

For UV irradiation, the medium was removed from the culture dish. The cells were washed with PBS and irradiated with 30 J/$m^2$ UVC light. After irradiation, the original culture medium was added back to the cells.

Hydroxyurea was dissolved in $H_2O$ and used at a final concentration of 1.5 mM. MG132 was dissolved in DMSO and used at a final concentration of 20 µM. Cycloheximide was dissolved in $H_2O$ and used at a final concentration of 100 µg/ml. G418 was dissolved in complete growth medium and used at a final concentration of 500 mg/ml. Etoposide was dissolved in DMSO and used at a final concentration of 50 µM. Methylmethansulfonate was dissolved in $H_2O$ and used at a final concentration of 5 mM.

*2.2.1.3 Transfection of cells*

H1299 cells were transfected by DNA-calcium phosphate co-precipitation. By this method, DNA is complexed with the calcium phosphate. The crystals precipitate on the cells and are taken up by the cells by endocytosis.

All solutions were prepared with ultrapure water and sterile equipment. First the required amount of DNA was mixed with the appropriate amount of water. Then $CaCl_2$ was added to a final concentration of 0.125 M. 2X HEPES-buffered saline (280 mM NaCl, 1.5 mM $Na_2HPO_4$, 50 mM HEPES pH 7.05) was added to a final concentration of 1X under constant mixing. Afterwards, the cell suspension was mixed with the transfection mix, transferred to a culture dish and incubated overnight. After 24 hours, the transfection medium was removed and 15% glycerol in PBS was added. The glycerol solution was incubated for 3 minutes. The cells were washed with PBS before complete culture medium was added to the cells.

MCF7 cells were transfected with ScreenFect® according to the manufacturer's recommendation. 6 µl transfection reagent were diluted with 150 µl dilution buffer and incubated 2-5 min at room temperature. Then 150 ng DNA were mixed with 150 µl dilution buffer. Thereafter, the diluted DNA was mixed with the diluted transfection reagent and incubated for 20 minutes at room temperature. During the incubation time, cells were trypsinized and counted. $5x10^5$ cells were seeded in 24-well plates. The DNA/transfection mixture was pipetted onto the cells and the cells were incubated for 48 hours.

*2.2.1.4 Infection of cells with lentiviruses*

1.6 x $10^4$ RKO cells were seeded in a well of a 96-well plate. The next day, the culture medium was replaced with 110 µl of normal growth medium. Lentiviruses were added at 0.5, 1, 2 and 5 multiplicity of infection (MOI). The next day, the

medium was replaced with 100 µl of fresh normal growth medium. The next day, puromycin was added to a final concentration of 0.5 µg/ml. Surviving cells were expanded with complete growth medium containing puromycin.

*2.2.1.5 Cell proliferation assay*

1 x $10^4$ cells were seeded in a well of a 24-well plate in triplicates and incubated under normal growth conditions. 0, 24, 48 and 72 hours after seeding, cells were counted. Therefore, cells were trypsinized with 0.5 ml trypsin. Trypsinization was stopped by addition of 2 ml complete growth medium. 10 µl of the cell suspension were pipetted into a Neubauer counting chamber and counted.

*2.2.1.6 Colony forming assay*

The colony forming assay was used to measure the clonogenic cell survival. This assay is based on the ability of a single cell to undergo several successive divisions (at least 5-6) and grow into a colony. H1299 cells were transfected with plasmids encoding Mdm2 and p53 together with a plasmid encoding FAM83F or with the empty vector pCDNA3, 48 hours after transfection, fresh growth medium was added and the cells were treated with G418 (500 mg/ml final concentration) for 2 weeks. Then the medium was aspirated and the colonies were fixed in 1% methanol for 20 minutes at room temperature. The colonies were visualized by staining with crystal violet blue for 15 minutes at room temperature. The cells were washed with PBS until the excess dye was removed. The colonies were counted by using the image-J program.

### 2.2.2 Protein Methods

#### *2.2.2.1 Immunofluorescence staining*

For immunofluorescence staining, cells were plated onto autoclaved cover slips. 48 hours after transfection, the medium was removed, the cells were washed with PBS and incubated with ice cold acetone/methanol (1:1) for 8 minutes on ice. After washing with PBS, the cells were blocked for 30 minutes at room temperature with blocking solution (1% goat serum, 1% bovine serum albumin in PBS). The primary antibody solution containing the DO1 anti-p53 antibody (mouse, monoclonal) diluted 1:500 and the anti-FAM83F antibody (rabbit polyclonal) diluted 1:500 was added and incubated overnight at 4°C. The samples were washed 3 times for 5 minutes with PBS. Then the secondary antibody solution containing the Alexa-488 coupled anti-rabbit antibody, diluted 1:500, and the Alexa-546 coupled anti-mouse antibody, diluted 1:500, was added together with Draq5 and diluted 1:1000. After 1 hour incubation in the dark at room temperature, the cells were washed three times with PBS and mounted onto microscope slides with Aqua-Poly/Mount. The slides were dried overnight and analysed with a Leica confocal microscope.

#### *2.2.2.2 SDS-PAGE and Western Blotting*

##### 2.2.2.2.1 Preparation of cell lysates

The culture medium was aspirated from the cells, the cells were washed with cold PBS and scraped from the culture dish. The cell suspension was transferred to a reaction tube and the cells were pelleted by centrifugation at 1200 rpm for 5 minutes. The cell pellet was suspended in 1% NP40 lysis buffer (50 mM Tis pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM PMSF) and incubated for 10 minutes on ice. The lysate was cleared by centrifugation at 1300 for 10 minutes at 4°C. The supernatant was transferred to a fresh reaction tube and the protein concentration was determined.

2.2.2.2.2 Bradford assay

The Bradford assay was used to determine the protein concentration. Therefore, the Roti®-Quant protein quantitation assay solution was diluted 1:5. Next, a standard curve was established. Therefore, 0, 2, 4 and 6 µl of a BSA solution (1 mg/ml) were mixed with 500 µl of the Roti®-Quant protein quantitation Assay solution. Then 2 µl of the cell lysate was also mixed with 500 µl with the Roti®-Quant protein quantitation Assay solution. 150 µl of these mixtures were pipetted into the wells of a 96-well plate and the absorbance was measured at λ595 nm. The standard curve was used to determine the concentration of the protein lysates.

2.2.2.2.3 Separation of proteins by SDS-PAGE

Cellular proteins can be separated on the basis of their mass by electrophoresis. For SDS-polyacrylamide gel electrophoresis, gel solutions were poured between two glass plates, a longer and a shorter one that were separated by spacers and fixed in a casting stand. First, the separating gel, that separates the proteins according to size, was poured. Therefore a 10% acrylamide gel solution (10% acrylamide, 375 mM Tris pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED) was prepared and poured between the two glass plates. 300 µl ethanol were carefully pipetted over the separating gel and the gel was allowed to polymerize. Then the ethanol was removed and a 5% stacking gel solution (5% acrylamide, 125 mM Tris pH 6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED) was prepared and poured over the separating gel. A comb was inserted to allow the formation of pockets into which the samples could be loaded later. After polymerization of the stacking gel, the glass plates with the gel were inserted into an electrophoreses chamber that was filled with running buffer (193 mM glycine, 24 mM Tris pH 6.8, 0.1% SDS) and electrophoresis was carried out at 120V.

2.2.2.2.4 Western Blotting and Immunedetection

After separating the protein samples by SDS-PAGE, they were transferred to a polyvinolidene fluoride membrane. Therefore, the membrane was first rinsed with methanol and then equilibrated with transfer buffer (0.193 M Glycine, 0.043 M Tris base, 10% methanol). Four pieces of filter paper and two transfer sponges were also soaked in transfer buffer. The membrane and gel was assembled in the blotting sandwich so that blot and membrane are in the centre. On each side of blot and membrane were two filter papers and a transfer sponge. All air bubbles were removed and the sandwich was placed into the transfer chamber. Thereby the side with the gel was located at the side of the cathode so that the negatively loaded proteins move towards the membrane. The chamber was filled with transfer buffer and the transfer was performed at 30V overnight.

The next day, the membrane was blocked in blocking solution (5% milk powder in PBS/0.02% Tween-20) for 1 hour. After blocking, the membrane was incubated over night at 4°C with a specific primary antibody. The next day, the membrane was washed three times with PBS/0.02% Tween-20 for 5 min each. Afterwards, the membrane was incubated with a horseradish peroxidase-coupled secondary antibody diluted 1:2000 in DMSO/10% FCS four 1 hour at room temperature. Thereafter, the Western blot was developed. A mixture of equal volumes of ECL (enhancer of chemioluminenscence) solution I (2.5 mM luminol, 0.396 mM coumaric acid, 225 mM Tris pH 8.5) and ECL solution II (0.0192% $H_2O_2$, 225 mM Tris pH 8.5) was poured over the membrane and incubated for 5 seconds. Thereafter, the membrane was wrapped into plastic foil and exposed against an X-ray film.

*2.2.2.3 Immunoprecipitation*

Cells were washed with ice-cold PBS and lysed with cold 0.5% NP-40 lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF) and were incubated for 15 min on ice. The lysates were centrifuged at 13.000 rpm for 15

min at 4°C. The supernatant was transferred into a fresh tube. 5% of the protein lysate were kept for input control. The remaining cellular lysate was incubated with the desired antibody coupled to a mixture of protein A-sepharose and protein G-sepharose (4:1) overnight at 4°C with end-over-end rotation. Thereafter, the sepharose was washed 3 times with 0.5% NP-40 buffer. After the last wash were the precipitates suspended in 2X sample buffer, vortexed and heated for 10 min to 95°C. The samples were stored at -20°C.

*2.2.2.4 Co-Immunoprecipitation*

For co-immunoprecipitations, the cells were lysed in cold CoIP-buffer (50 mM Tris pH8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF) for 15 min on ice. The lysates were cleared by centrifugation at 13.000 rpm for 15 min at 4°C. The supernatant was transferred into a fresh tube and pre-cleared with 50 µl protein A-sepharose for 1 h at 4°C with end-over-end rotation. Thereafter, 5% of the protein lysate was kept for input control. The desired antibody was coupled to protein A sepharose, added to the remaining cellular lysate and incubated overnight at 4°C with end-over-end rotation. The next day, the sepharose was washed 3 times with CoIP-buffer. After the last wash were the precipitates suspended in 2X sample buffer, vortexed and heated for 10 min to 95°C. The samples were stored at -20°C.

*2.2.2.5 GST-Pulldown Assay*

The GST-Fam83F fusion protein was purified from a culture of E.coli BL21 cells that had been transformed with a plasmid encoding the gene for it. The bacteria were grown in LB medium (10 g/l Tryptone, 5 g/l Yeast extract, 10 g/l NaCl) overnight at 37°C with shaking. The next morning, the overnight culture was diluted with LB medium and IPTG was added to a final concentration of 100 µM. After culturing the bacteria for 6 h at room temperature with constant vigorous shaking, the bacteria were collected by centrifugation at 6,000 rpm for 15 min at 4°C and stored at -80°C until they were further processed.

The cell pellet was lysed in lysis buffer (1% Triton-X-100, 1 mM PMSF in PBS), briefly sonicated and incubated in ice for 30 min. The insoluble material was removed by centrifugation at 10,000 rpm at 4°C for 30 min. Thereafter, the clarified lysate was mixed with 500 µl glutathione-sepharose for 4 h at 4°C by end-over-end rotation. The sepharose was washed 6 times with lysis buffer and the GST-protein was eluted with GST-elution buffer (10 mM reduced glutathione, 50 mM Tris pH8). The expression of the purified protein was tested by SDS-PAGE.

10 µg of the eluted GST-Fam83F were mixed with 10 µg His-tagged p53, also expressed in bacteria and purified, and diluted with pulldown buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.5% Triton-X-100). 10% of the mixture was taken for input control. To the rest of the lysate were 30 µl glutathione-sepharose added and the mixture was incubated overnight at 4°C with end-over-end rotation. The next day, the sepharose was washed 3 times with pulldown-buffer. After the last wash were the precipitates suspended in 2X sample buffer, vortexed, heated for 10 min to 95°C and further analysed by Western Blotting.

*2.2.2.6 Cell Fractionation*

Cells were washed with ice-cold PBS, scraped from the dish and collected by centrifugation at 2000 rpm for 5 min. Then the pellet was washed in 400 µl cold buffer A (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EGTA and 0.1 mM EDTA). The supernatant was removed and the pellet was suspended in 400 µl fresh buffer A and incubated for 15 min on ice to allow swelling of the cells. 25 µl of a 10% solution of NP-40 were added, the sample was vortexed vigorously and centrifuged for 1 min at 13,000 rpm. The supernatant, containing the cytoplasmic fraction was transferred into a new tube. The pellet, containing the nuclei, was suspended in 150 µl cold buffer C (20 mM HEPES pH7.5, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol) and incubated on ice for 20 min with periodical vigorous vortexing. The nuclear lysate was collected by centrifugation for 5 min at 13,000 rpm. Cytoplasmic and nuclear fractions were used immediately for SDS-PAGE or stored at -20°C for short time intervals.

*2.2.2.7 Ubiquitination assay*

H1299 cells were transfected with plasmids encoding His-tagged ubiquitin, Mdm2 and p53 together with a plasmid encoding Fam83F or with vector DNA. 24 h after transfection, cells were treated for 6 h with 20 μM MG132 to increase the amount of ubiquitinated proteins.

The cells were washed with ice cold PBS and suspended in PBS. 10% of the cell suspension was kept for input control and lysed in NP-40 buffer as described above. The remaining cells were collected by centrifugation for 5 min at 2,000 rpm and lysed in guanidinium lysis buffer pH8 (6 M guanidinium HCl, 0.1 M $Na_2HPO_4/NaH_2PO_4$ pH8, 0.01 M Tris pH8, 5 mM imidazole, 10 mM β-mercaptoethanol). $Ni^{2+}$-nitrilotriacetic acid (Ni-NTA)-agarose was added to the lysate and the mixture was incubated overnight at room temperature with end-over-end rotation. The next day, the agarose was pelleted by centrifugation at 2,000 rpm for 5 min followed by washing. The agarose was subsequently washed with guanidinium buffer, urea buffer pH8 (8 M urea, 0.1 M $Na_2HPO_4/NaH_2PO_4$ pH8, 0.01 M Tris pH8, 10 mM β-mercaptoethanol), buffer A pH6.3 (8 M urea, 0.1 M $Na_2HPO_4/NaH_2PO_4$ pH6.3, 0.01 M Tris pH6.3, 10 mM β-mercaptoethanol), buffer A plus 0.2% Triton-X-100 and with buffer A plus 0.1% Triton-X-100. The ubiqutinated proteins were eluted with elution buffer (60 mM imidazole. 5% SDS, 0.15 M Tris pH6.8, 30% glycerol, 0.72 M β-mercaptoethanol). The eluate was mixed with 2x SDS-PAGE (1:1), vortexed, heated to 95°C for 5 min and loaded onto a SDS-PAGE gel.

*2.2.2.8 Luciferase Assay*

Cells were seeded into a 96-well plate and transfected with 50 ng of a plasmid encoding the p53-dependent reporter PG13 and with 5 ng of a plasmid encoding renilla luciferase together with a plasmid encoding Fam83F or with empty vector. 48 h after transfection, cells were lysed in passive lysis buffer (Promega) and

transferred to -80° for 4 hours. The luminometer (Perkin Elmer, VictorLight 1420) was programmed for the appropriate delay and measurement times.

20 µl of the cell extract was pipetted into a well of a white 96-well plate. The plate was placed into the luminometer. For renilla activity, the injector added 100 µl of renilla substrate (1 µM coelenterazine, 0.5 M NaCl, 1 mM EDTA, 0.1 M potassium phosphate buffer pH8) per well and the well was read immediately. For measuring Firefly activity added the injector 70 µl of firefly solution I (1 mM ATP, 1mM DTT, 25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA) and 20 µl of the firefly substrate solution (200 µM luciferin, 25 mM Gly-Gly, 15 mM $MgSO_4$, 4 mM EGTA). The reaction was measured immediately. Reporter activity was calculated by dividing the readings for the firefly luciferase by the readings for renilla luciferase.

### 2.2.3 Nucleic acid methods

#### *2.2.3.1 Total RNA extraction*

For the preparation of total RNA was the Quick-RNATM Mini-Prep (Plus) kit (Zymo Research, Germany) used.

H1299 cells were pelleted by centrifugation at 500 g for 1 minute. The supernatant was removed and the cell pellet was suspended in RNA lysis buffer. The lysate was transferred into a Spin-AwayTM Filter in a collection tube and the sample was centrifuged. Then 1 volume ethanol was added to the flow through, mixed well and transferred to a Zymo-SpinTM IIICG column in a collection tube. The sample was centrifuged again and the flow through was discarded. 700 µl wash buffer were added to the column followed by centrifugation and removal of the flow through. Then 400 µl RNA wash buffer were added to the column followed by centrifugation for 2 minutes to ensure complete removal of the wash buffer. Thereafter, the column was transferred into an RNAse-free tube. 100 µl RNAse-free water were added to the column matrix and centrifuged. The eluted RNA was used immediately or stored at -80°C.

*2.2.3.2 cDNA synthesis*

For cDNA synthesis was the total mRNA reversely transcribed. At first was the RNA for 1 h at 37°C incubated with the RNAse inhibitor Ribo-BlockTM and with DNAse I. After DNA digestion, 1 µl of 25 mM EDTA was added to a 20 µl reaction and the sample was heated to 65°C for 10 min. Thereafter, 1 µl of a 200 ng/µl solution of random primers was added and the mixture was incubated at 70°C for 5 min. Then 1 µl of the MLVRT (Revert AidTM H Minus MMuLV) was added together with 2 µl of a 10 mM solution of dNTPs, 4 µl of a 5x reaction buffer and 4 µl $H_2O$. The mixture was incubated at 25°C for 10 min followed by 42°C for 60 min and 70°C for 10 min. The cDNA was diluted with $H_2O$ and stored at -20°C.

*2.2.3.3 Polymerase Chain Reaction for quality control*

After preparing the cDNA, the quality was checked by polymerase chain reaction (PCR) using primers for the housekeeping genes (β-Actin). For the PCR, 0.4 µM forward and reverse primers, 0.2 mM dNTPs, 2 µl of a 10x GoTaq polymerase buffer and 2 µl GoTaq polymerase were mixed with 4 µl of the cDNA. The PCR program was set to 95°C for 3 min. Then 30 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 1 min were performed, followed by 10 min at 72°C. The product was then analysed by gel electrophoresis.

*2.2.3.4 Agarose gel electrophoresis*

Agarose gel electrophoresis was used to separate DNA fragments. The concentration of agarose tanged from 1% to 2% depending on the size of the fragments.

The agarose was dissolved by boiling in 1x TAE buffer (40 mM Tris pH7.2, 20 mM sodium acetate, 1 mM EDTA). The solution was cooled down and ethidium bromide was added to a final concentration of 0.4 µg/ml. Thereafter, the solution was poured into a horizontal gel chamber and a comb was inserted to allow the formation of

slots. After the gel was polymerized, the comb was removed and the gel was transferred to an electrophoresis chamber that was filled with 1x TAE buffer. The samples were mixed with DNA sample buffer (Peqlab) and pipetted into the slots. Electrophoresis was performed at 80-120 V, depending on the size of the gel. The DNA fragments were visualized with UV-light.

*2.2.3.5 qRT-PCR*

Quantitative real time PCR (qRT-PCR) was used to determine the relative amounts of RNAs.

4 µl of the cDNA solution were mixed with 10 µl 2x SYBR Green, 1 µl of gene-specific forward and reverse primers (50 ng/µl) and 4 µl nuclease-free $H_2O$. qRT-PCR was performed in duplicates. A control was also performed where the template was replaced with $H_2O$. The reaction was performed in the ABI StepOnePlus System (Applied Biosystems) with the following program: 95°C for 15 sec followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The reaction was finished by 15 sec at 95°C, 1 min at 60°C and 15sec at 95°C. The housekeeping gene β-actin was used for normalization.

*2.2.3.6 Sub-cloning of Fam83F*

The coding sequence of Flag-Fam83F-Δ1, Flag-Fam83F-Δ2 and GST-Fam83F was amplified from the plasmid encoding full length wild-type Fam83F. The forward primer for Flag-Fam83F-Δ1 and Flag-Fam83F-Δ2 contained a BamHI and the reverse primer a NotI restriction site while the forward primer for GST- Fam83F contained a BamHI and the reverse primer an XhoI restriction site.

2.2.3.6.1 Amplification of the insert

For the amplification of the insert were 30 cycles of a PCR reaction performed. The reaction was done twice to increase the amount of the PCR product. The PCR reaction contained 1 µl of template DNA, 1 µl of the Prime Star DNA polymerase, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 4 µl of a 10x Taq buffer and 0.4 µM of the forward and reverse primers. The PCR program was set to 95°C for 2 min and 30 cycles of 95°C for 30 sec, 58°C for 1 min and 72°C for 1 min followed by 5 min at 72°C.

2.2.3.6.2 Restriction digest

The PCR reaction was loaded onto an agarose gel and separated by electrophoresis. The Fam83F fragment was excised from the gel and purified using the PeqGold gel extraction kit. The purified product was digested with BamH1 and NotI in the case of Flag-Fam83F-Δ1, Flag-Fam83F-Δ2. The pcDNA3 vector into which the fragments shall be cloned was digested with the same enzymes. The fragment for GST-Fam83F was digested with BamH1 and XhoI and the pGEX-4T-2 vector was digested with the same enzymes. Therefore, the PCR fragments and vectors were mixed with 1 unit of the restriction enzymes per 1 µg of DNA and the appropriate reaction buffer. The digest was performed for 1 h at 37°C. After the digest, the DNA fragments and vectors were separated by agarose gel electrophoresis, excised from the gel and purified.

2.2.3.6.3 Purification of DNA by agarose gel electrophoresis

Gel purification starts with agarose gel electrophoresis that separates DNA by size. After electrophoresis, DNA bands were visualized under UV light. The DNA was excised, transferred to a 1.5 ml reaction tube and weighted. The purification was done by using the PeqGold Gel extraction Kit according to the manufacturer's recommendation. At first, 100 µl binding buffer was added to 100 µg of the weighted

agarose piece with the DNA and incubated at 60°C with vortexing until the agarose was melted. The solution was transferred to a purification column that was connected to a collection tube. The sample was centrifuged for 1 min at 2,000 rpm. The flow through was discarded and the column was washed once with 300 µl binding buffer followed by washing twice with 500 µl washing buffer. The column was centrifuged to remove any residual buffer. For elution was the column transferred into a new 1.5 ml tube. 30 µl elution buffer were added to the column, incubated for 5 min and centrifuged for 1 min at 2,000 rpm.

2.2.3.6.4 Ligation of DNA fragments

All ligations were performed with a T4 DNA ligase. Therefore, two different ratios of insert DNA:vector DNA and a negative control without insert were adjusted to 17 µl with $H_2O$. The sample was mixed with 2 µl 10x ligase buffer and 1 µl T4 ligase and incubated overnight at 16°C.

2.2.3.6.5 Transformation of bacteria

Competent bacteria were transformed with each of the ligation reactions. Therefore, 100 µl of chemically competent E. coli were incubated with 20 µl of the ligation reaction for 30 min on ice followed by a heat-shock at 42°C for 50 sec. Then the mix was incubated on ice for 2 min. Thereafter, 900 µl LB medium were added to the mix and incubated at 37°C for 1 hour with shaking. In the meantime, an agar plate (LB medium, 1 g/l agarose and 100 µg/ml ampicillin) was warmed to room temperature. The bacteria were pelleted by centrifugation for 1 min at 8,000 rpm. 800 µl of the supernatant were aspirated, the bacteria were suspended in the remaining 200 µl LB medium, spread over the agar plate and incubated overnight at 37°C. Positive clones were further amplified.

2.2.3.6.6 Purification of plasmids

Plasmids were purified with the Qiagen plasmid purification kit® according to the manufacturer's recommendation. This protocol is based on a modified alkaline lysis procedure followed by binding of the plasmid DNA to a resin. Low molecular weight impurities, proteins, RNA and dyes are removed and the plasmid DNA is eluted in a high salt buffer. The plasmid is then desalted and concentrated by isopropanol precipitation.

Plasmid preparation starts with the picking of a single colony and inoculation of a 2-5 ml starter culture in LB medium containing the appropriate selective antibiotic that is incubated overnight at 37°C with shaking. The starter culture is then diluted 1/500 with LB medium containing the appropriate antibiotic and incubated overnight at 37°C with shaking. The bacteria were harvested by centrifugation at 6,0000 x g for 15 min at 4°C. The bacterial pellet was suspended in 4 ml buffer P1. Then 4 ml buffer P2 were added and carefully mixed by inverting the reaction tube. 4 ml cold buffer P3 were added and mixed by inverting the reaction tube. The mixture was incubated for 15 min on ice and centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was applied onto a Qiagen column that had been equilibrated with 4 ml buffer QBT. The column was washed with 5 ml QC and the DNA was eluted with 4 ml of buffer QF. The DNA was precipitated by adding 3.5 ml isopropanol and collected by centrifugation at 9,000 rpm for 30 min at 4°C. The DNA was washed with 70% ethanol and dissolved in TE buffer (10 mM EDTA, 50 mM Tris pH8).

2.2.3.6.7 Determination of the nucleic acid concentration

The concentration of nucleic acids was determined by measuring the optical density (OD). 2 µl of the nucleic acid were applied onto the Nano-Drop® and analysed with the ND-1000 software version 3.1.2. A ratio of OD260/OD280 of 1.8 for DNA (or 2.0 for RNA) indicates a nucleic acid preparation that is free if protein contamination. A ratio of OD260/OD230 above 1.6 indicates that the solution is free of organic chemicals and solvents.

# 3. Results

## 3.1 Fam83F increases p53 levels

The first observation that Fam83F is a regulator of p53 was made by Ping Zhang who identified Fam83F when he was screening a cDNA library for evolutionary conserved regulators of p53 (Zhang et al., 2015). In order to confirm his initial finding, I overexpressed human Fam83F together with p53 and Mdm2 in the p53-negative cell line H1299. I then monitored the abundance of p53, Mdm2 and Fam83F by Western Blotting.

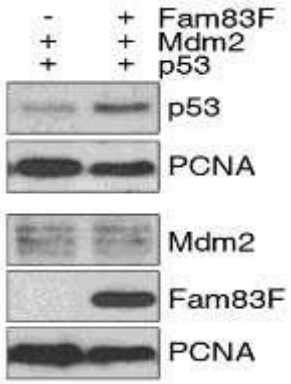


**Figure 3.1: Overexpression of Fam83F increases p53 abundance.** H1299 cells were transfected with plasmids encoding Mdm2 and p53 together with a plasmid encoding Fam83F or with vector DNA for control. Abundance of p53, Mdm2 and Fam83F was determined by Western Blotting. Abundance of PCNA (proliferating cell nuclear antigen) was determined for loading control.

As shown in figure 3.1, overexpression of Fam83F indeed increased the abundance of p53. At the same time, there was no obvious increase in the abundance of Mdm2 (Figure 3.1).

p53 is a short-lived protein that is rapidly degraded after its synthesis (Boehme and Blattner, 2009). Since Mdm2 is a major regulator of the stability of the p53 protein (Haupt et al., 1997; Kubbutat et al., 1997), I wondered whether the induction of p53

by Fam83F depends on the presence of Mdm2. To address this question, I transfected H1299 cells with plasmids encoding p53 and Fam83F together with a plasmid encoding Mdm2, or with vector DNA.

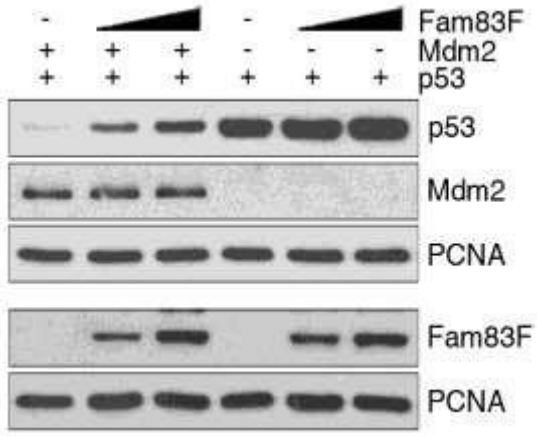


**Figure 3.2: Induction of p53 by Fam83F requires the presence of Mdm2.** H1299 cells were transfected with an increasing amount of a plasmid encoding Fam83F together with plasmids encoding p53 and Mdm2, or with vector DNA. Abundance of p53, Mdm2, Fam83F and PCNA, for loading control, was determined by Western Blotting.

As shown in Figure 3.2, the increase in p53 abundance caused by Fam83F can only be seen in the presence of co-transfected Mdm2. This increase in p53 abundance, moreover, correlated strongly with the amount of the plasmid that encodes Fam83F that has been transfected (Figure 3.2).

Regulation of protein stability is a common mechanism by which abundance and activity of proteins is controlled. This is also the case for p53 (Ashcroft et al., 1999, Ashcroft and Vousden, 1999).

Since the regulation of p53 by Fam83F depended on Mdm2, I assumed that Fam83F might regulate the abundance of p53 by altering its rate of degradation. To address this, I transfected H1299 cells with plasmids encoding p53 and Mdm2 together with a plasmid encoding Fam83F. Twenty-four hours after transfection, I treated the cells

with cycloheximide, an inhibitor of protein biosynthesis (Baliga et al., 1969), and harvested cells every fifteen minutes. I then monitored the abundance of p53 by Western Blotting.

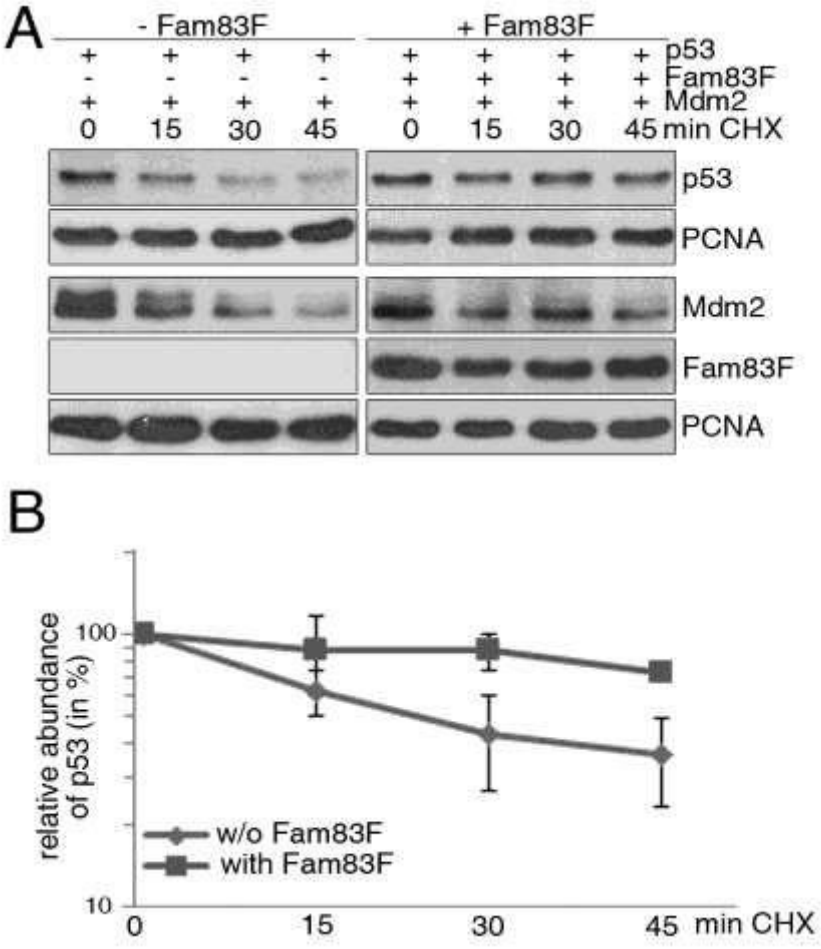


**Figure 3.3: Fam83F enhances the half-life of p53. (A)** H1299 cells were transfected with plasmids encoding p53 and Mdm2 and with a plasmid encoding Fam83F or with vector DNA. 24 h after transfection, cycloheximide (CHX) were added to a final concentration of 100 µg/ml. Cells were harvested at 0, 15, 30 and 45 min after addition of cycloheximide and the abundance of p53 was determined by Western Blotting. **(B)** The signals for p53 and PCNA were quantified and the relative abundance of p53 was calculated and blotted. The graph shows mean values and standard deviations from 3 independent experiments. The relative amount of p53 at time "0" was set to 100 %.

As shown in figure 3.3, p53 abundance decreased rabidly when Fam83F was not over-expressed. However, when Fam83F was over-expressed, degradation of p53

was almost completely prevented (Figure 3.3 A). This can also be seen in the graph which also shows a strong extension of the half-life of the p53 protein in the presence of over-expressed Fam83F (Figure 3.3 B). This result shows that Fam83F increases p53 protein stability.

In addition to protein stability, the rate of transcription and also the stability of the RNA determine how much of a protein is finally available in the cell. Therefore, although I could already show that Fam83F regulates protein stability, I wondered whether Fam83F also affects the amount of p53 RNA. In order to investigate this, I transfected H1299 cells with plasmids encoding p53 and Mdm2 together with a plasmid encoding Fam83F or with vector DNA.

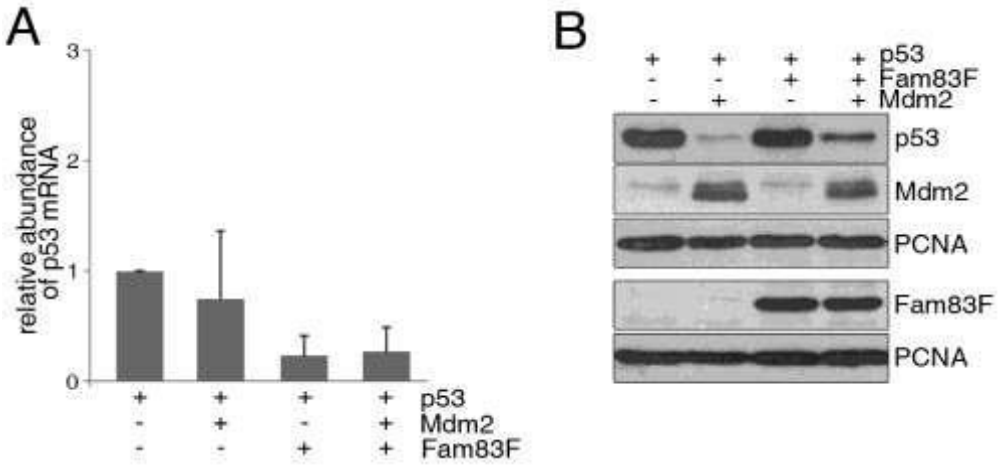


**Figure 3.4: Fam83F does not induce p53 RNA.** H1299 cells were transfected with the indicated combinations of plasmids encoding p53, Mdm2 and Fam83F. 24 h after transfection, cells were harvested and divided into two parts. **(A)** One part of the cells was lysed, RNA was prepared and the relative abundance of p53 RNA was determined by qRT-PCR. The graph shows mean values and standard deviations of 3 independent experiments. The amount of p53 RNA in cells that had only been transfected with p53 and vector DNA was set to 1. **(B)** The second part of the cells was used to monitor expression of p53, Mdm2 and Fam83F by Western Blotting.

Twenty-four hours after transfection, I harvested the cells and divided them into two parts. From one part, I purified the RNA and performed qRT-PCR. The second part I used to monitor the abundance of p53, Mdm2 and Fam83F by Western Blotting.

Although over-expression of Fam83F increased the abundance of p53 at the protein level, it did not increase the amount of p53 RNA (Figure 3.4).

Although different degradation routes have been described for p53 (Tsvetkov et al., 2010; Chao, 2015), the majority of p53 molecules are degraded by 26S proteasomes (Love et al., 2013). Since Fam83F increased p53 abundance by reducing its protein degradation, I wondered whether it is the proteasomal degradation pathway that is inhibited by Fam83F. To address this question, I over-expressed Fam83F in H1299 cells together with p53 and Mdm2. Twenty-four hours after transfection, I treated a part of the cells with MG132, a peptide aldehyde that blocks the proteolytic activity of the 26S proteasome (Myung et al., 2001). For control, I treated the cells with DMSO, the solvent of MG132.

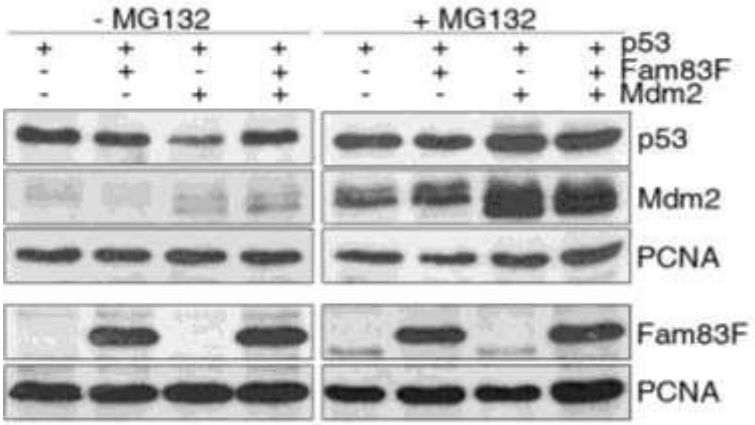


**Figure 3.5: Fam83F increases p53 abundance only in the presence of functional proteasomes.** H1299 cells were transfected with plasmids encoding p53 together with plasmids encoding Fam83F or Mdm2 or both. 24 h after transfection, MG132 (10 μM f.c.) or DMSO, for control, were added. 16 h after addition of MG132, cells were harvested and the abundance of p53, Mdm2 and Fam83F were determined by Western Blotting. Abundance of PCNA was determined for loading control.

As shown in figure 3.5, the abundance of p53 was not affected by Fam83F when the cells were treated with MG132. In contrast, when the cells were only treated with the solvent, the influence of Fam83F on p53 abundance could be clearly observed (Figure 3.5). This result strongly suggests that Fam83F blocks the degradation of p53 in 26S proteasomes.

## 3.2 Fam83F associates with p53

Since Fam83F induces p53 abundance and since p53 levels are tightly controlled by Mdm2 (Haupt et al., 1997; Kubbutat et al., 1997), I wondered whether Fam83F might associate with p53 or Mdm2. Therefore, I transfected H1299 cells with a plasmid encoding Flag-tagged Fam83F and with a plasmid encoding p53. To see whether Fam83F associates with Mdm2 or eventually even requires the presence of both, p53 and Mdm2 at the same time for binding, I also transfected a plasmid encoding Mdm2 or vector DNA. I immuneprecipitated Fam83F and monitored associated p53 and Mdm2 abundance by Western Blotting.

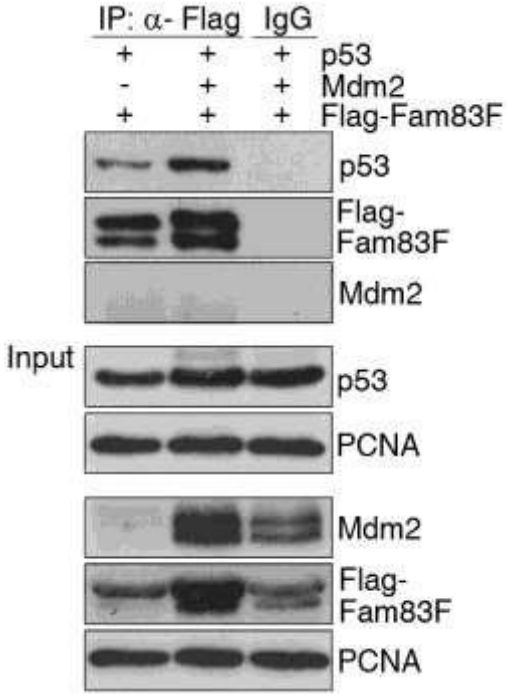


**Figure 3.6: Fam83F interacts with p53.** H1299 cells were transfected with plasmids encoding Flag-tagged Fam83F and p53 together with a plasmid encoding Mdm2 or with vector DNA. 24 h after transfection, cells were lysed, Flag-tagged Fam83F was immune-precipitated and the associated p53 and Mdm2 were monitored by Western Blotting. Precipitation with IgG was performed for control. An aliquot of the lysed cells was used to monitor the abundance of p53, Mdm2 and Flag-tagged Fam83F (Input). Hybridization with an antibody targeted against PCNA was performed for loading control.

As shown in figure 3.6, p53 associated with Fam83F, this interaction was further enhanced when Mdm2 was present (Figure 3.6). In contrast, Mdm2 did not co-precipitate with Fam83F (Figure 3.6).

After having determined the association between p53 and Fam83F after overexpression, I wondered whether the two proteins might also interact with each other under physiologic conditions. For testing this, I immuneprecipitated Fam83F from RKO cells. As p53 is usually present in limited amounts in the cells, I treated the cells with hydroxyurea (HU) in order to increase p53 abundance. After immune-precipitation, I monitored the associated p53 by Western Blotting.

As shown in figure 3.7, p53 associated with Fam83F also when the proteins are not overexpressed, which confirms the previous result (Figure 3.7, Figure 3.6).

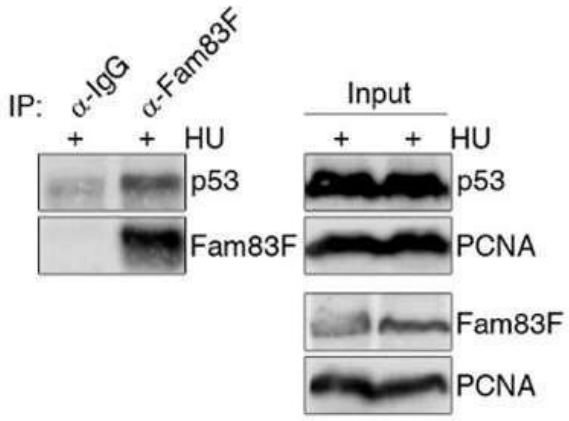


**Figure 3.7: Fam83F interacts with p53 in RKO cells.** RKO cells were treated with 1.5 mM hydroxyurea (HU). 12 h after HU addition, cells were lysed and Fam83F was immune-precipitated. The associated p53 was monitored by Western Blotting. Precipitation with IgG was performed for control. An aliquot of the lysed cells was used to monitor the abundance of p53 and Fam83F (Input). Hybridization with an antibody targeted against PCNA was performed for loading control.

Since I found that Fam83F associates with p53, I wondered whether this interaction is direct or whether it may require additional proteins.

To test this, I performed a GST-pulldown assay. I expressed His-tagged p53 and GST-tagged Fam83F in bacteria, purified these proteins and mixed them in a test tube. I then precipitated Fam83F using glutathione-sepharose and monitored the associated p53 by Western Blotting.

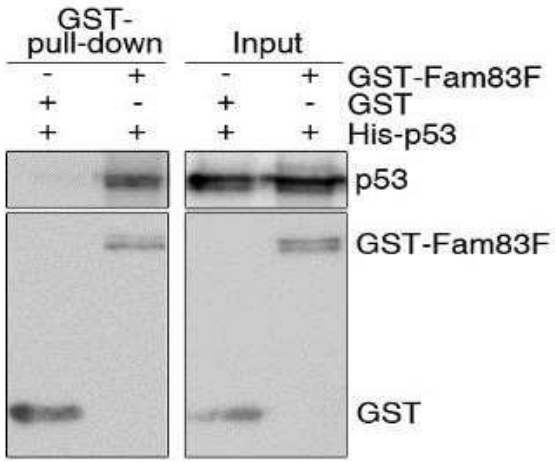


**Figure 3.8: Fam83F interacts directly with p53.** His-tagged p53 was mixed with GST-tagged Fam83F. An aliquot of the mix was taken to monitor abundance of the two proteins (Input). From the remaining mixture, GST-Fam83F was precipitated by coupling to glutathione sepharose and the associated p53 was monitored by Western blotting.

As shown in figure 3.8, His-tagged p53 co-precipitated with the GST-tagged Fam83F indicating that both proteins interact directly with each other (Figure 3.8).

p53 and Mdm2 interact strongly with each other and this strong interaction is required for the degradation of p53 in 26S proteasomes (Haupt et al., 1997; Kubbutat et al., 1997). Fam83F furthermore bound p53 directly (Figure 3.8). Since Fam83F increases p53 abundance by preventing its degradation in 26S proteasomes, I

wondered whether Fam83F might interfere with the interaction of p53 with Mdm2. To address this, I transfected H1299 cells with plasmids encoding p53 and Mdm2. In addition, I transfected a plasmid encoding Fam83F or vector DNA, for control. 24 h after transfection, I immune-precipitated Mdm2 and monitored associated p53 (and Fam83F) by Western Blotting. Precipitation with IgG was performed for control.

As shown in figure 3.9, Fam83F did not reduce the interaction between p53 and Mdm2. In fact, the interaction of p53 and Mdm2 was even enhanced in the presence of Fam83F, probably because of the higher amounts of p53 (Figure 3.9). This experiment furthermore confirms the previous result that Fam83F does not bind to Mdm2 (Figure 3.6).

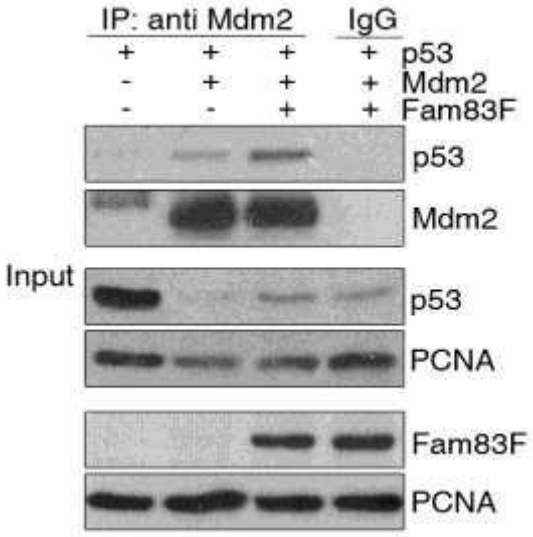


**Figure 3.9: Fam83F does not interfere with the interaction of p53 and Mdm2.** H1299 cells were transfected with plasmids encoding p53, Mdm2 and Fam83F as indicated. 24h after transfection, cells were harvested. An aliquot of the lysate was used to monitor the expression of p53 and Fam83F by Western blotting (Input). From the remaining cells, Mdm2 was immune-precipitated and associated p53 and Fam83F were detected by Western Blotting. Precipitation with IgG was performed for control.

After having found that Fam83F and p53 co-precipitate, I determined the domains of the two proteins that interact. Therefore, I transfected H1299 cells with a plasmid expressing Flag-tagged Fam83F together with plasmids expressing V5-tagged full length p53, the p53 deletion mutant Δ1, comprising amino acid one to three hundred

or the deletion mutant Δ2, comprising amino acid one to one hundred-fifty and monitored associated p53 by Western Blotting.

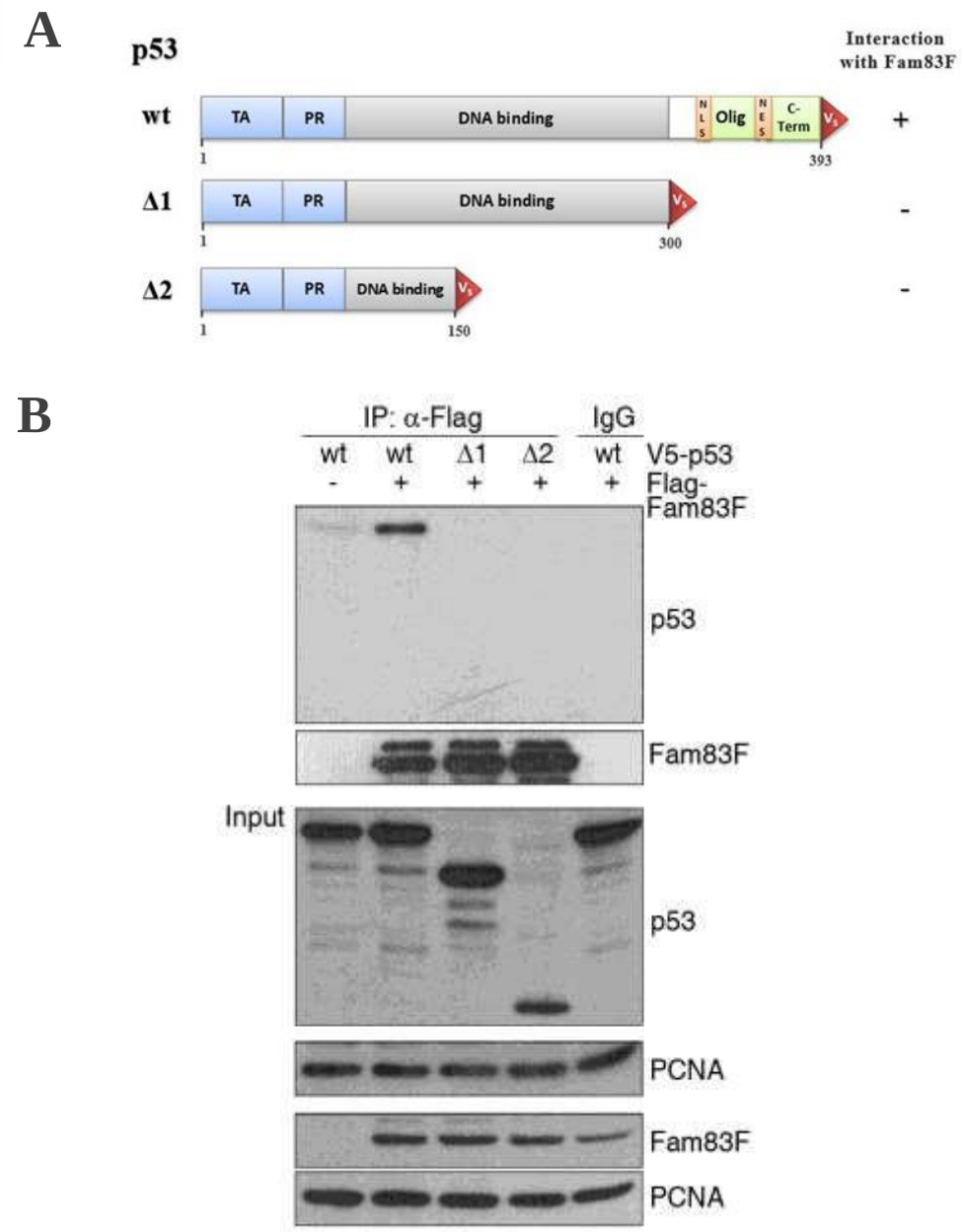


**Figure 3.10: Fam83F associates with the C-terminus of p53. (A)** Cartoon of the employed constructs of p53 and their interaction with Fam83F. **(B)** H1299 cells were transfected with plasmids encoding Flag-tagged Fam83F and the indicated V5-tagged full length (wt) or mutant forms of p53 (Δ1: 1-300 aa; Δ2: 1-150 aa). 24 h after transfection, cells were lysed, Flag-tagged Fam83F was immune-precipitated and the associated p53 was monitored by Western Blotting. Precipitation with IgG was performed for control. An aliquot of the lysed cells was used to monitor abundance of p53 and Flag-Fam83F by Western Blotting (Input). Hybridization with an antibody targeted against PCNA was performed for loading control.

As shown in figure 3.10, only the full length p53 protein but not the deletion mutants interacted with Fam83F. This result indicates that the interaction with Fam83F involves the last 100 amino acids of p53.

After having determined the domain of p53 which is involved in the interaction with Fam83F, I mapped the area on Fam83F that interacts with p53. Therefore, I transfected H1299 cells with a plasmid expressing p53 together with a plasmid expressing Flag-tagged full length Fam83F or the Flag-tagged deletion mutant Δ1, comprising amino acid one to three hundred and ninety-seven or the Flag-tagged deletion mutant Δ2, comprising amino acid one to two hundred and eighty-three. I immuneprecipitated Flag-tagged Fam83F and monitored the associated p53 by Western Blotting.

As shown in figure 3.11, p53 associated with both deletion mutants of Fam83F as well as with wild type Fam83F, indicating that the interaction with p53 occurs via the N-terminal domain of Fam83F (Figure 3.11).

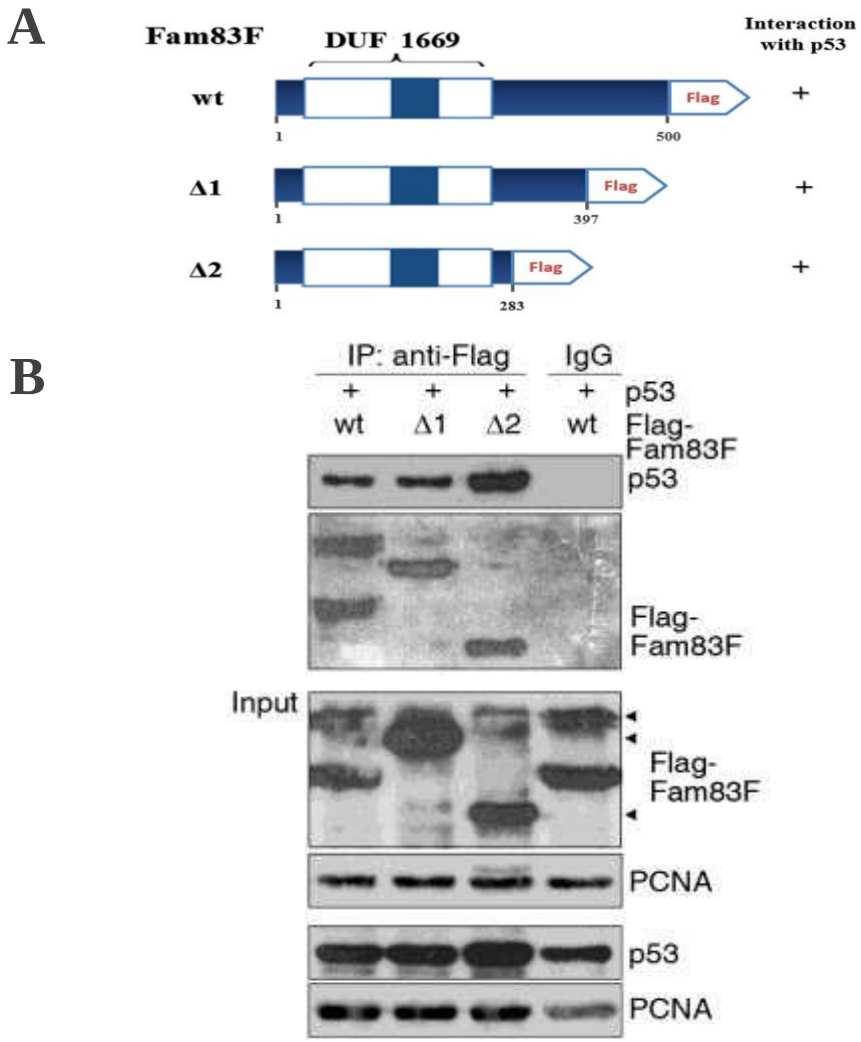


**Figure 3.11: The N-terminus of Fam83F associates with p53. (A)** Cartoon of the employed constructs of Fam83F and their interaction with p53. **(B)** H1299 cells were transfected with plasmids encoding the indicated full length (wt) or mutated forms of Flag-tagged Fam83F (Δ1: 1-397 aa; Δ2; 1-283 aa) together with a plasmid encoding p53. 24 h after transfection, cells were lysed, Flag- tagged wild type Fam83F or the Fam83F deletion mutants were immune-precipitated and the associated p53 was monitored by Western Blotting. Precipitation with IgG was performed for control. An aliquot of the lysed cells was used to monitor abundance of p53 and Flag-Fam83F (Input). Hybridization with an antibody targeted against PCNA was performed for loading control.

Since the N-terminus of Fam83F was sufficient for the interaction of Fam83F with p53, I wondered whether this domain might also be sufficient for regulating p53 abundance. To address this, I transfected H1299 cells with plasmids expressing p53 and Mdm2 together with a plasmid that either expressed full length Flag-tagged

Fam83F or Flag-tagged deletion mutants of Fam83F. I then monitored the abundance of p53, Mdm2 and Fam83F by Western Blotting.

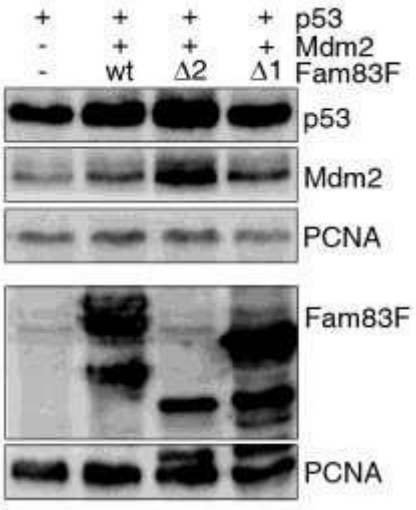


**Figure 3.12: The N-terminus of Fam83F is necessary for inducing p53 abundance.** H1299 cells were transfected with plasmids encoding p53, Mdm2 and the indicated full length (wt) and partially deleted forms of Fam83F (Δ1: 1-397aa and Δ2: 1-283aa). 24 h after transfection, cells were lysed, and abundance of p53, MDM2 and Fam83F were determined by Western Blotting. Abundance of PCNA was determined for loading control.

As shown in figure 3.12, the first 397 amino acids of Fam83F are sufficient for stabilizing p53. Most surprisingly, when further 114 amino acids were removed from the C-terminus (Δ2; 1-283aa), induction of p53 was even stronger (Figure 3.12). Overall, these data strongly imply that the domain that is responsible for the induction of p53 resides in the N-terminal half of the Fam83F protein, the area that also interacts with p53 (Figure 3.11).

## 3.3 Fam83F reduces p53 and Mdm2 ubiquitination

p53 is a protein that shuttles between the nucleus and the cytoplasm (Roth et al., 1998; Stommel et al., 1999). Since it was reported that nucleo-cytoplasmic shuttling of the p53 protein is essential for Mdm2-mediated degradation of the p53 protein (Roth et al., 1998), I thought to test whether Fam83F might eventually stabilize p53 by sequestering the protein in the nucleus.

To address this question, I transfected H1299 cells with plasmids expressing Mdm2 and p53 together with a plasmid that expresses Fam83F or with vector DNA. Twenty-four hours after transfection, I fractionated the cells into nuclear and cytoplasmic lysate and monitored the abundance of p53 and Fam83F by Western Blotting. To confirm the fractionation, I used GAPDH (glutaraldehyde phosphatdyl dehydrogenase) as a cytoplasmic marker and Histone H3 as a nuclear marker.

In consistency with published data (Rotter et al., 1983), I found the majority of p53 in the nucleus and only a minority in the cytoplasm (Figure 3.13). Overexpression of Fam83F did not change this pattern, indicating that Fam83F has no influence on the subcellular localization of p53. Surprisingly, in the presence of Fam83F and absence of Mdm2, I found more p53 protein in the cytoplasm (Figure 3.13). Of not, in consistency with data provided by genecards (www.genecards.org), Fam83F was present both in the cytoplasm and nucleus and thus in the same compartments as p53 (Figure 3.13), which is also a prerequisite for the observed interaction of Fam83F and p53 (Figure 3.6). Hybridization of the Western Blots with antibodies targeted against GAPDH and Histone H3 show that the fractionation into the nuclear and cytoplasmic compartment was successful.

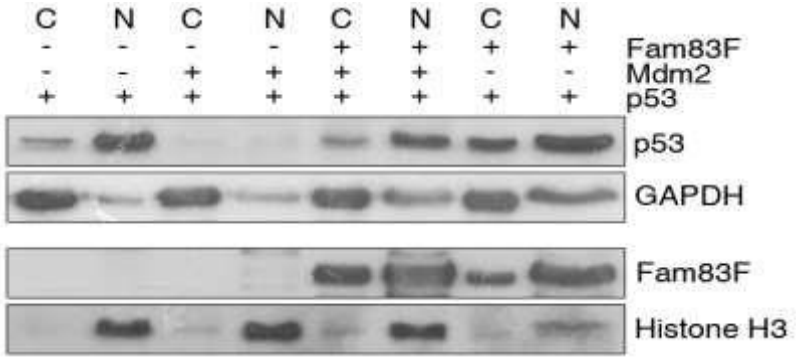


**Figure 3.13: Fam83F does not affect the sub-cellular distribution of p53.** H1299 cells were transfected with plasmids encoding p53, Mdm2 and Fam83F as indicated. Cells were fractionated into cytoplasmic and nuclear lysates and abundance of p53 and Fam83F was determined by Western Blotting. Hybridization with antibodies targeted against GAPDH and Histone-H3 were performed for control (C: cytoplasmic fraction; N: nuclear fraction).

For confirming the result of the cell fractionation, I performed immune- fluorescence staining. Therefore, I cultured H1299 cells on gelatine-coated glass slices. Then, I transfected the cells with plasmids encoding p53 and Mdm2 together with a plasmid encoding Fam83F or with empty vector. Forty-eight hours after transfection, I fixed the cells and incubated them with antibodies targeted against p53 and Fam83F. To visualize the nuclei, I incubated the cells with Draq5, a fluorescent dye that binds to DNA.

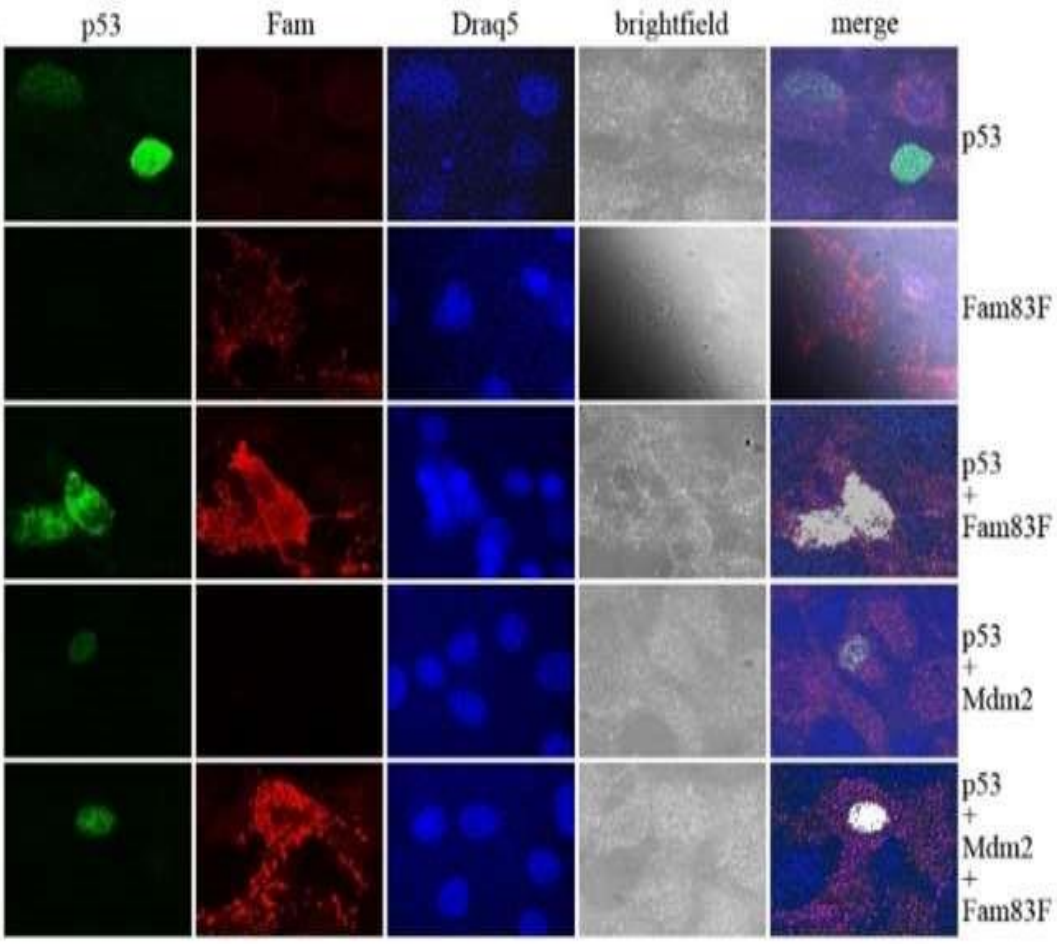


**Figure 3.14: Fam83F and p53 are present in the same subcellular compartment.** H1299 cells were cultured on gelatine-coated glass slides and transfected with plasmids encoding p53 and Mdm2 together with a plasmid encoding Fam83F or with vector DNA. 48 h after transfection, cells were fixed with ice cold acetone-methanol and incubated with the anti-p53 antibody DO-1 (green) and anti-Fam83F (red). After washing, cells were incubated with Alexa-fluor 448- and Alexa-fluor 546-coupled anti-mouse and anti-rabbit antibodies together with Draq5 (blue). Thereafter, the slides were washed, mounted onto microscope slides and analysed with a Leica LSM microscope.

In agreement with the fractionation, the immunofluorescence staining showed that that p53 is predominantly located in the nucleus and largely excluded from the cytoplasm (Figure 3.14). The overexpression of Mdm2 resulted in a decrease in the intensity of the p53 signal. Fam83F was present in both, the cytoplasm and the nucleus. The overexpression of Fam83F did not alter the localization of p53 but resulted in an increase in the signal for p53 (Figure 3.14) which is consistent with the data obtained by Western Blotting (Figure 3.1).

Degradation of p53 in 26S proteasomes requires prior ubiquitination (Fang et al., 2000; Love et al., 2013). Since the N-terminus of Fam83F interacts with the C-terminus of p53, which holds the main ubiquitination sites of p53 (Rodriguez et al., 2000; Lohrum et al., 2001), I wondered whether Fam83F might stabilize p53 by inhibiting its ubiquitination. To investigate this, I transfected H1299 cells with plasmids encoding p53, Mdm2 and Fam83F together with a plasmid encoding His-tagged ubiquitin. Twenty-four hours after transfection, I treated the cells with MG132 to stabilize the p53 protein also in the absence of Fam83F. This way,

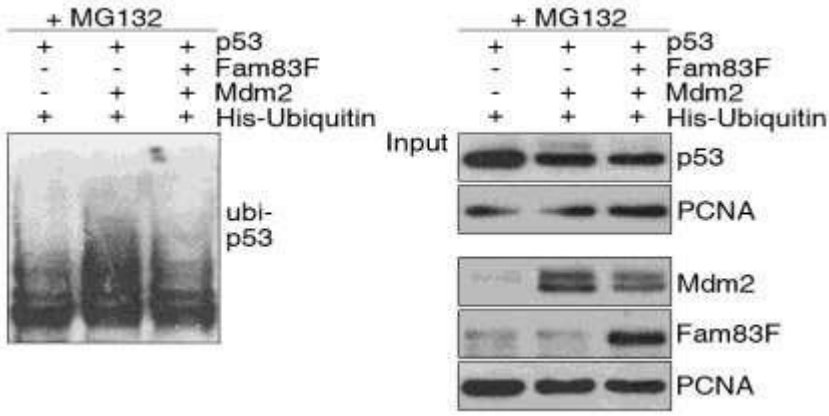


**Figure 3.15: Ubiquitination of p53 is reduced by overexpressed Fam83F.** H1299 cells were co-transfected with plasmids encoding p53, His-tagged ubiquitin, Mdm2 and Fam83F in the indicated combinations. 24 h after transfection, cells were treated with 10 μM MG132 for 8 hours. Then cells were harvested. An aliquot of the cells was lysed in NP40 buffer and expression of p53, Mdm2 and Fam83F was determined by Western Blotting (Input). The remaining cells were lysed in guanidinium lysis buffer and ubiquitinated proteins were collected by adsorption to $Ni^{2+}$ agarose. Ubiquitinated protein were eluted and separated by SDS-PAGE. Abundance of p53 was detected by Western Blotting.

it was possible to get similar amounts of the p53 protein both in the absence and in the presence of Fam83F that made it possible to compare the level of ubiquitination. Eight hours after addition of MG132, I harvested the cells and purified the ubiquitinated proteins by adsorption to $Ni^{2+}$-agarose. I separated the ubiquitinated proteins by SDS-PAGE and monitored the amount of ubiquitinated p53 by Western Blotting. As shown in figure 3.15, overexpression of Fam83F reduced p53 ubiquitination.

Mdm2 is the main master regulator for p53 (Momand et al., 2000) and auto-ubiquitination of Mdm2 has been reported to promote p53 ubiquitination (Ranaweera and Yang, 2013). This raised the question whether Fam83F might reduce p53 ubiquitination because it interferes with Mdm2 auto-ubiquitination. To address this question, I transfected H1299 cells with plasmids encoding Mdm2 and Fam83F together with a plasmid encoding His-tagged ubiquitin. Twenty-four hours after

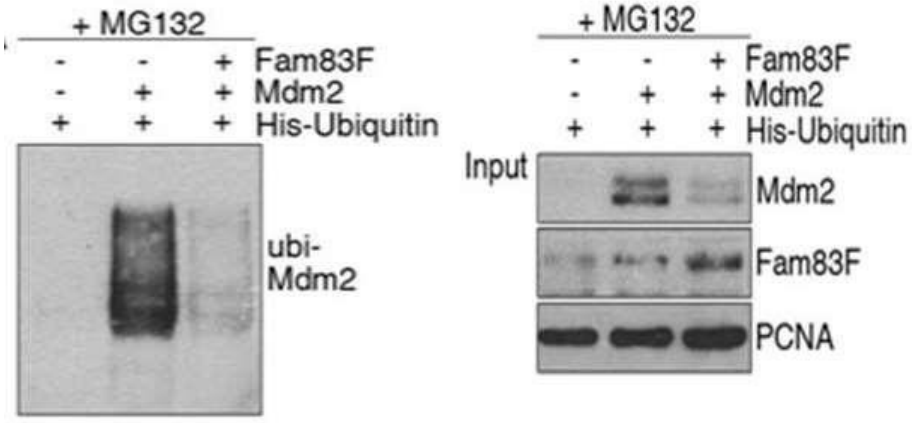


**Figure 3.16: Ubiquitination of Mdm2 is reduced in the presence of Fam83F.** H1299 cells were transfected with plasmids encoding His-tagged ubiquitin, Mdm2 and Fam83F. 24 h after transfection, cells were treated with 10 μM MG132 for 8 hours. Then the cells were harvested. An aliquot of the cells was lysed in NP40 buffer and expression of Mdm2 and Fam83F was determined by Western Blotting (Input). The remaining cells were lysed in guanidinium lysis buffer and ubiquitinated proteins were collected by adsorption to $Ni^{2+}$-agarose. Ubiquitinated proteins were eluted and separated by SDS-PAGE. Abundance of Mdm2 was determined by Western Blotting.

transfection, I treated the cells with MG132 for eight hours. I harvested the cells, purified the ubiquitinated proteins by adsorption to $Ni^{2+}$-agarose, separated the ubiquitinated proteins by SDS-PAGE and monitored the amount of ubiquitinated Mdm2 by Western Blotting.

As shown in figure 3.16, similar to ubiquitination of p53, ubiquitination of Mdm2 was strongly reduced after overexpression of Fam83F (Figure 3.16).

## 3.4 Fam83F enhances the transcriptional activity of p53

To investigate whether the increase in p53 abundance by Fam83F is also reflected by an increase in p53 activity, I performed a reporter assay. For this, I transfected MCF7 cells, which express wild type p53, with a plasmid encoding Fam83F together

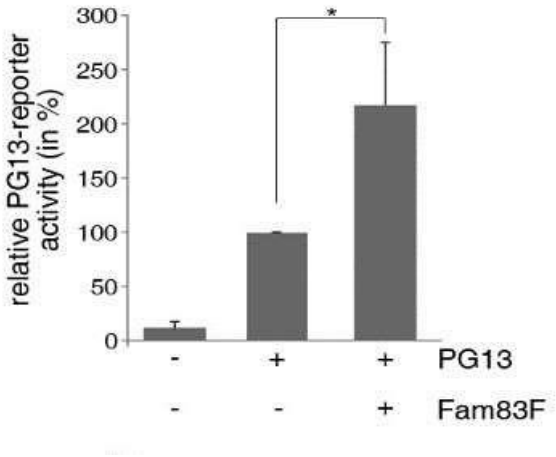


**Figure 3.17: Fam83F increases p53 activity.** MCF7 cells were transfected with the p53- dependent reporter PG13 and with a plasmid encoding renilla luciferase, for transfection control, together with a plasmid encoding Fam83F or with vector DNA. 48 h after transfection, cells were lysed and firefly- and renilla–luciferase activity was determined. Relative firefly activity was calculated by normalization with the readings for renilla luciferase. The value of cells that had been transfected only with plasmids encoding the reporter was set to 1. The graph shows mean values and standard deviations of three independent experiments. The *p*-value was calculated by the two-tailed T test. *$p<0.05$.

with a plasmid encoding the p53-responsive reporter PG13 that possesses 13 repeats of the consensus p53-binding site fused to the gene for the firefly luciferase (El-Deiry et al., 1993) and a plasmid encoding the renilla luciferase for internal control. Forty-eight hours after transfection, I measured the firefly and renilla luciferase activity and calculated the relative activity of p53.

As shown in figure 3.17, there was a significant increase in p53 activity after overexpression of Fam83F, indicating that the increase in p53 abundance caused by Fam83F translates into more active p53.

To further investigate the influence of Fam83F on p53 activity, I downregulated Fam83F in RKO cells by infection with a lentivirus containing a shRNA that was directed against Fam83F. After selection of Fam83F knockdown clones, I monitored the expression of Fam83F by Western blotting.

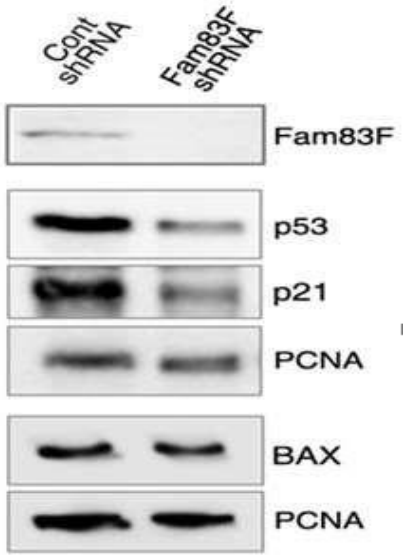


**Figure 3.18: Knockdown of Fam83F.** RKO cells have been infected with lentiviruses carrying a shRNA directed against Fam83F or a control shRNA and the infected cells have been selected with 0.5 µg/ml puromycin. Afterwards, the cells were analysed for the abundance of Fam83F, p53, p21 and Bax, and for PCNA for loading control, by Western Blotting.

As shown in figure 3.18, the expression of fam83F was clearly reduced after infection with lentiviruses carrying a shRNA targeted against Fam83F. Knockdown of Fam83F furthermore reduced the steady-state level of p53 in RKO cells (Figure 3.18). Since *p21* and *bax* are target genes of p53 (El-Deiry et al., 1994; Miyashita et al., 1994), I also tested the levels of p21 and Bax. As shown in figure 3.18, p21 levels were strongly reduced after downregulation of Fam83F while bax levels were no affected (Figure 3.18).

One of the major activities of p53 is arresting the cell cycle (Agarwal et al., 1995; Shaw, 1996). Thus, if Fam83F indeed controls the activity of p53 then it should influence cell proliferation. In order to test this prediction, I monitored the proliferation of the RKO cells with downregulated Fam83F. Therefore, I plated a defined number of cells into the wells of a 24-well plate and counted the cells every day.

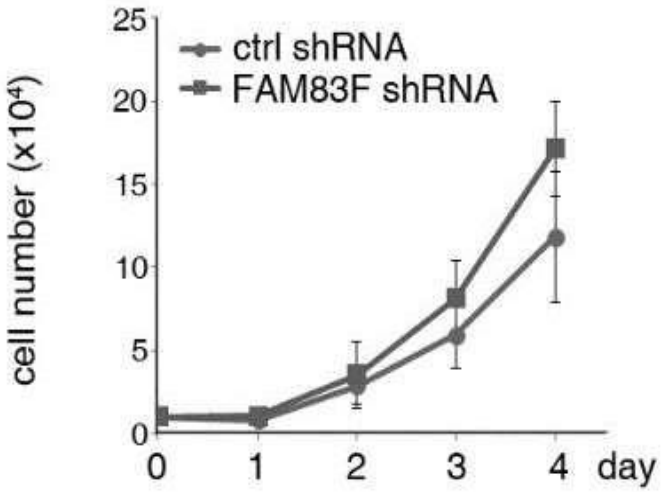


**Figure 3.19: Knockdown of Fam83F increases cell proliferation.** RKO cells with downregulated Fam83F or the corresponding control cells with normal levels of Fam83F were plated at a density of $10^4$ cells per well into a 24 well plate and counted every day. The graph shows mean values and standard deviations of three independent experiments.

As shown in figure 3.19, knocking-down Fam83F enhanced the proliferation of RKO cells (Figure 3.19).

## Results

To confirm the result of the proliferation assay with downregulated Fam83F, I performed a clonogenic assay with overexpressed Fam83F. Therefore, I transfected H1299 cells with plasmids encoding p53 and Mdm2 together with a plasmid encoding Fam83F or with vector DNA. For control I also transfected cells just with vector DNA. Twenty-four hours after transfection, I treated the cells with G418 to select for the transfected cells. Within the next two weeks, I allowed the transfected cells to form colonies. To visualize the colonies, I fixed them with methanol and stained them with crystal violet. I then analysed the result with the Image-J program.

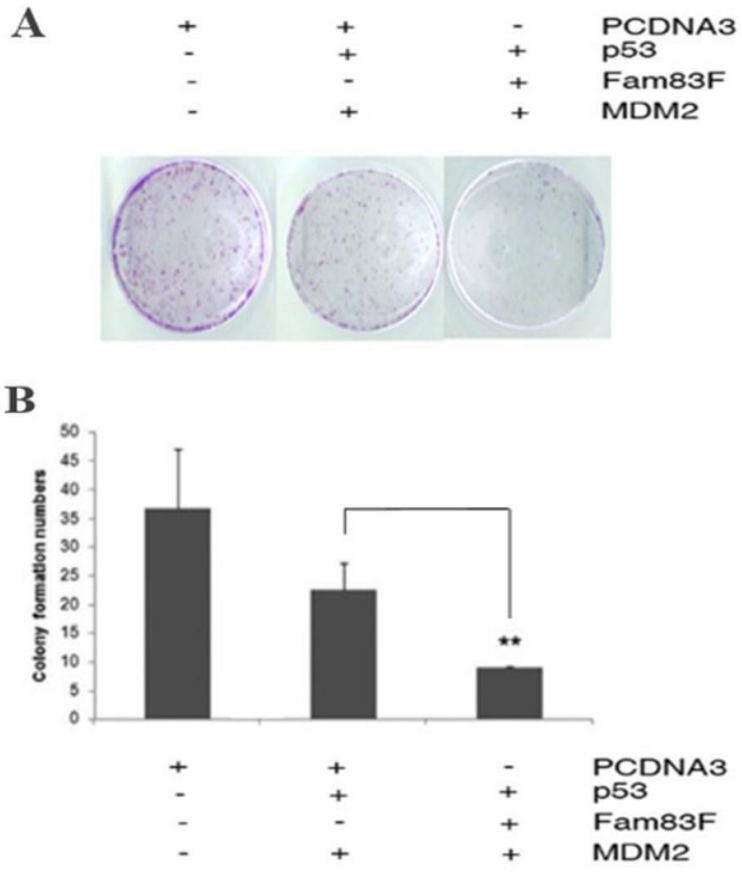


**Figure 3.20: Overexpression of Fam83F inhibits cell proliferation. (A)** H1299 cells were transfected with plasmids encoding Mdm2 and p53 together with a plasmid encoding Fam83F or with vector DNA. 24 h after transfection, the cells were treated with G418 (500 mg/ml) for 2 weeks. The colonies were visualized by staining with crystal violet and counted with the Image-J program. **(B)** The graphs shows mean values and standard deviations of the colony numbers of three independent experiments. ** $P \leq 0.01$.

Consistent with earlier findings (Casey et al., 1991; Isaacs et al., 1991), overexpression of p53 reduced the number and size of the colonies (Figure 3.20). Importantly, overexpression of Fam83F resulted in a further reduction of the number of colonies (Figure 3.20).

## 3.5 Fam83F is induced in response to DNA damage

p53 is strongly induced and activated in response to DNA damage (Lakin and Jackson, 1999; Hirao et al., 2000). Since Fam83F significantly increases p53 abundance and activity, I wondered whether Fam83F might also be induced by DNA damage and thus contribute to the activation of p53 in response to DNA damage. To address this, I incubated human fibroblasts with 50 μM etoposide. Etoposide forms a ternary complex with DNA and inhibits topoisomerase II (Walles et al., 1996). This inhibition of topoisomerase II leads to the generation of DNA double strand breaks. After addition of etoposide to the human fibroblasts, I harvested the cells after increasing time points and monitored the abundance of Fam83F, p53 and Mdm2 by Western blotting.

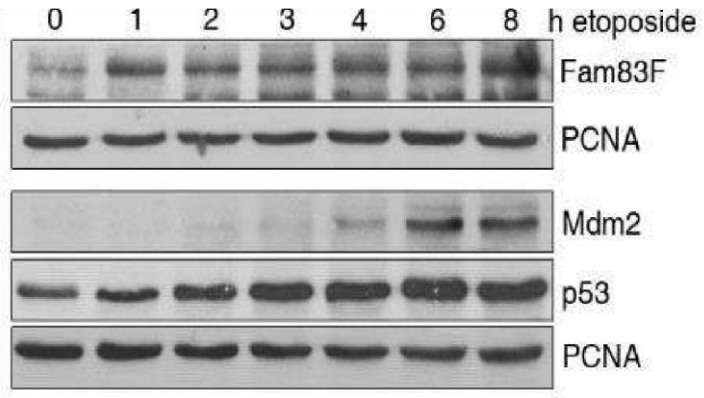


**Figure 3.21: Fam83F is induced in response to etoposide.** Primary human fibroblasts (GM38) were treated with 50 μM etoposide for the indicated time. The cells were harvested and the abundance of Fam83F, p53 and Mdm2 was determined by Western Blotting. Hybridization with an antibody targeted against PCNA was performed for loading control.

As shown in figure 3.21, Fam83F levels were induced by etoposide. The increase in Fam83F protein levels was already visible at one hour after addition of etoposide. With increasing time Fam83F levels were further increased (Figure 3.21). Induction of p53 could also be observed already at one hour after etoposide addition and p53 also accumulated further with time. In fact, p53 and Fam83F showed similar kinetics of induction. Mdm2, a transcriptional target of p53, was induced later and accumulated from four hours after etoposide addition onwards until the end of the experiment (Figure 3.21).

To confirm and extent the result with etoposide, I treated the primary fibroblasts also with DNA damaging agents that induce other kinds of lesions. To this end, I used methyl-methansulfonate (MMS; Lundin et al., 2005), a compound that alkylates the bases of the DNA, ultraviolet light (UVC; Goto et al., 2015) that induces thymidine dimers and 4-6-photoproducts in the DNA, and hydroxyurea that oxidizes and depurinates the DNA (Sakano et al., 2001). For MMS and etoposide treatment, I added the drug and harvested the cells at increasing times after drug addition. For UVC-treatment, I removed the medium from the cells, washed the cells with PBS, exposed them to UVC light, added the original medium back to the cells and harvested the cells after increasing times. For the treatment with hydroxyurea, I treated the RKO cells with hydroxyurea for seventeen hours. Then I removed the medium and washed the cells excessively with PBS and harvested the cells after increasing times.

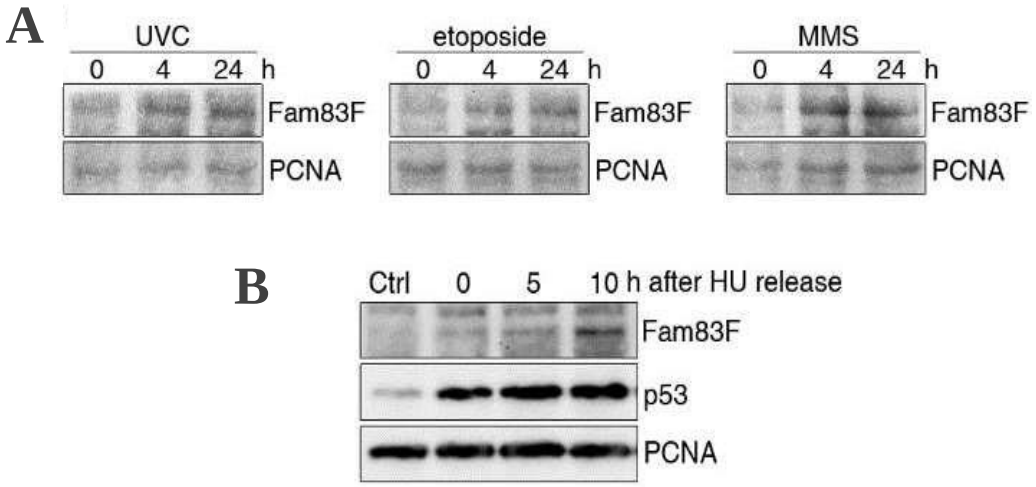


**Figure 3.22: Fam83F is induced in response to DNA damage. (A)** Primary human fibroblasts (GM38) cells were exposed to UVC light (30 J/m$^2$), Etoposide (50 mM) and methyl methanesulfonate (MMS; 5 mM). Cells were harvested at the indicated times and lysed. Abundance of Fam83F was monitored by Western Blotting. PCNA was used for loading control. **(B)** RKO cells were treated with 1.5 mM hydroxyurea (HU) for 17 hours. Then the HU was removed and cells were washed with PBS and incubated with fresh medium. The cells were harvested at the indicated time points after HU release (ctrl: untreated cells). Abundance of Fam83F and p53 was monitored by Western Blotting. PCNA was used for loading control.

As shown in figure 3.22, Fam83F was induced in response to all adverse agents (Figure 3.22). The induction of Fam83F again mirrored the induction of p53 (Figure 3.21; Figure 3.22-B) although the induction of p53 appeared to be stronger which may be due to the higher amount of p53 available in the cells.

p53 is activated in response to DNA damage and induces p21, an inhibitor of cyclin-dependent kinases and cell cycle arrest (Ding et al., 2003). Since Fam83F induces p53 activity and is also upregulated in response to DNA damage, I wondered whether Fam83F might be required for the full activation of p53 in response to DNA damage. To answer this question, I employed the RKO cells that had been infected with lentiviruses to downregulate Fam83F (Figure 3.18). I irradiated these cells with UVC-light or treated them with etoposide or hydroxyurea and monitored the expression of p53 and p21.

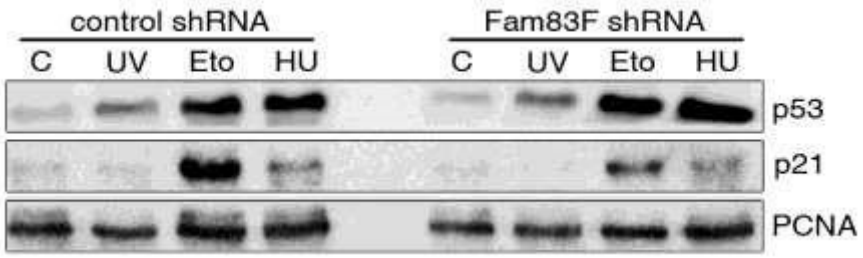


**Figure 3.23: Knockdown of Fam83F reduced p21 expression in response to DNA damage.** RKO cells that had been infected with a control shRNA and RKO cells that had been infected with a shRNA targeted against Fam83F were treated with hydroxyurea (HU; 1.5 mM) or etoposide (Eto; 20 mM), or irradiated with UVC (UV, 30 J/m$^2$). 12 hours after the treatment, cells were harvested. Abundance of p21 and p53 was monitored by Western Blotting. PCNA was used for loading control.

As shown in figure 3.23, downregulation of Fam83F reduced the expression of p21 after treatment with etoposide or hydroxyurea. In contrast to the treatment of the cells with etoposide or hydroxyurea, I could not detect induction of p21 after irradiation of the cells with UV-light. Surprisingly, I could not detect a decrease in the expression of p53 after DNA damage when Fam83F was downregulated (Figure 3.23).

## 3.6 Fam83F interacts with mutant p53

In a recent report, Fam83F was shown to be a target of microRNAs and downregulation of Fam83F by microRNAs resulted in tumour-inhibition (Mao et al., 2016; Yang et al., 2017). These findings are in contrast to my own results where Fam83F showed growth suppressive activities. One resolution to this discrepancy could be provided by the mutational status of p53. It is known since several years that p53 is frequently mutated in human cancer and mutations in p53 are a hallmark of oesophageal squamous cell carcinoma (Liang et al., 1995; Ireland et al., 2000). Most importantly, mutant p53 is not just an inactive protein. Instead, mutant p53 frequently gains oncogenic activities (Freed-Pastor and Prives, 2012). If Fam83F would also control mutant p53 then this activity could explain the above mentioned discrepancy. To investigate this, I first determined whether Fam83F and mutant p53

interact. Therefore, I transfected H1299 cells with a plasmid encoding wild-type p53 or a gain of function mutant of p53 (R273H or R175H) together with a plasmid encoding Flag-tagged Fam83F or with vector DNA. Twenty-four hours after transfection, cells were lysed, Flag-tagged Fam83F was immune-precipitated and the associated p53 was monitored by Western Blotting.

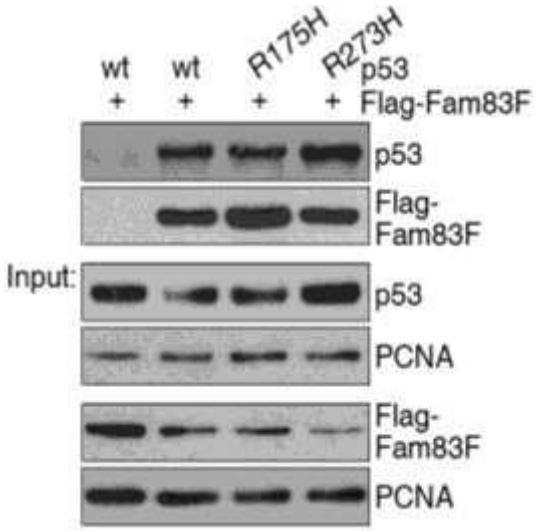


**Figure 3.24: Fam83F interacts with mutant p53.** H1299 cells were transfected with plasmids encoding wild-type (wt) p53 or the p53 mutants R175H or R273H together with a plasmid encoding Flag-tagged Fam83F or with vector DNA. 24 hours after transfection, cells were lysed, Flag-tagged Fam83F was immune-precipitated and the associated p53 was monitored by Western Blotting. An aliquot of the lysed cells was used to monitor abundance of p53 and Flag-Fam83F (Input). Hybridization with an antibody targeted against PCNA was performed for loading control.

As shown in figure 3.24, mutant p53 associated with Fam83F to the same extent as wild type p53 (Figure 3.24).

I then wondered whether Fam83F might influence the abundance of mutant p53. To investigate this I transfected H1299 cells with plasmids encoding wild-type or mutant p53 (R175H or R273H) together with plasmids encoding Mdm2 and Fam83F, or with vector for control. Twenty-four hours after transfection, I monitored p53 levels by Western Blotting. As shown in figure 3.25, overexpression of Fam83F did not affect the abundance of the p53 mutants R273H and R175H.

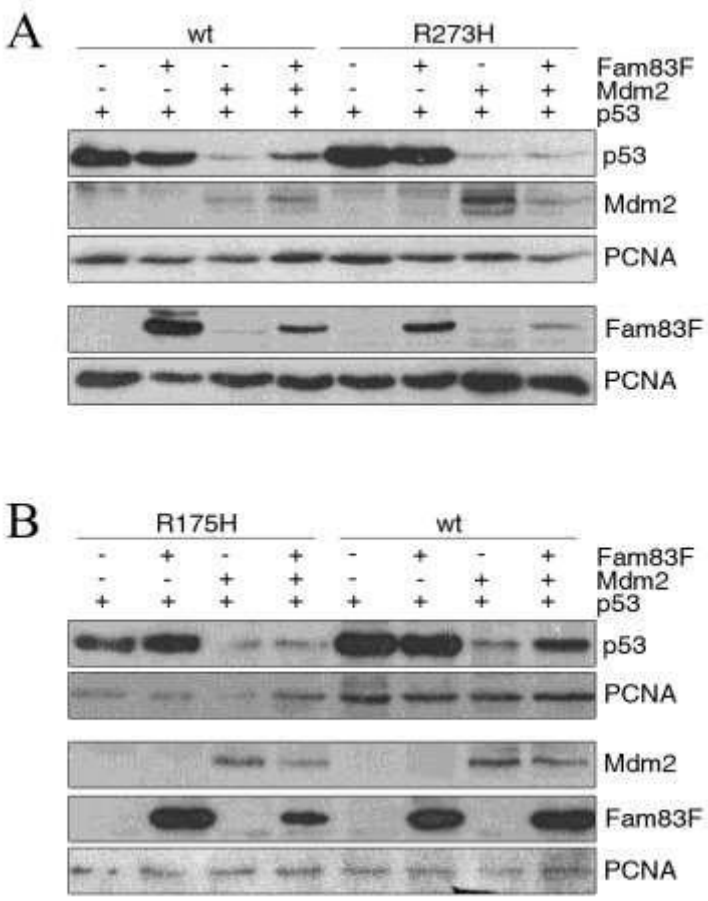


**Figure 3.25: Fam83F does not regulate the abundance of mutant p53. (A)** H1299 cells were transfected with plasmids encoding wild-type (wt) p53 or the p53 mutant R273H, Mdm2 and Fam83F in the indicated combinations. **(B)** H1299 cells were transfected with plasmids encoding wild type (wt) p53 or the p53 mutant p53 R175H, Mdm2 and Fam83F in the indicated combinations. Abundance of p53, Mdm2 and Fam83F was monitored by Western Blotting. Hybridization with an antibody targeted against PCNA was performed for loading control.

# 4. Discussion

Although p53 was initially regarded as an oncogene, it is known for several decades that p53 is frequently mutated in cancer (Takahashi et al., 1989). Moreover, cells lacking p53 have been shown to be cancer prone (Donehower et al., 1992). p53 is activated in response to various stresses and induces expression of different genes whose protein products mediate apoptosis, cell cycle arrest, senescence, autophagy, inhibition of angiogenesis and regulation of energy metabolism (Vogelstein et al., 2000; Beckerman and Prives, 2010). Under normal conditions, p53 is maintained at low levels, primarily by the oncoprotein Mdm2 that directly inhibits p53's transcriptional activity and that, as a RING-finger-containing ubiquitin ligase, targets p53 for proteasomal degradation (Momand et al., 1992; Chen et al., 1995; Haupt et al., 1997; Kubbutat et al., 1997). Mdm2 also mediates p53 nuclear export (Roth et al., 1998).

Although p53 is one of the most extensively studied proteins, much still remains to be elucidated; particularly as the p53 network is complex and many regulators that are involved in the p53 network are still unknown. In this study, I investigated the regulation of p53 by Fam83F, a novel regulator of p53 that was recently identified in our lab (Zhang et al., 2015).

## 4.1 Fam83F increases the abundance of p53

Fam83F is one of the genes that had been identified by screening a cDNA library from medaka (*Oryzias latipes*) for proteins that alter the abundance of p53 and or Mdm2 (Zhang et al., 2015). In this screen, Fam83F was found to increase p53 abundance with no effect on Mdm2 levels (Zhang et al., 2015). I confirmed this result using the human *fam83F* gene. The increase in Fam83F levels correlated strongly with the amount of transfected *fam83F*. However, the increase in p53 abundance was only seen when Mdm2 was also overexpressed. This result indicates that Fam83F regulates p53 abundance in an Mdm2-dependent manner. In contrast to

the protein, I did not find any evidence for a regulation of p53 RNA by Fam83F. Overexpression of *fam83F* even decreased the amount of p53 RNA. This result strongly implies that Fam83F regulates p53 at the protein level. The increase in p53 abundance was due to a strong reduction in its degradation. When I treated the cells with cycloheximide, I observed that overexpression of *fam83F* greatly extended the half-life of p53.

p53 abundance is primarily controlled by its protein stability and Mdm2-mediated ubiquitination and degradation is the main regulator for this process. I have observed that Fam83F stabilizes p53 by reducing its degradation in 26S proteasomes. As ubiquitination of p53 is a strong requirement for this process (Varshavsky, 1997), I expected that Fam83F may interfere with p53 ubiquitination. Indeed, overexpression of *fam83F* strongly reduced p53 ubiquitination. In addition, Fam83F also reduced Mdm2 auto-ubiquitination. Since auto-ubiquitination of Mdm2 promotes p53 polyubiquitination (Ranaweera and Yang, 2013), it is possible that Fam83F suppresses p53 ubiquitination by reducing Mdm2 auto-ubiquitination.

The reduction in p53 and Mdm2 ubiquitination after overexpression of fam83F implies that it may directly interact with these proteins. Indeed, I could show that the N-terminus of Fam83F associates with the C-terminus of p53. The six C- terminal lysine residues of p53 are the predominant sites for Mdm2 mediated ubiquitination (Lohrum et al., 2001). Thus, an alternative possibility for the reduced ubiquitination of p53 is that binding of Fam83F to the C-terminus of p53 might mask the ubiquitination sites in the C-terminus of p53. Ubiquitination and degradation of p53 requires a strong binding to Mdm2. Therefore, a further possibility how Fam83F could reduce p53 ubiquitination and degradation would be by interfering with the binding of p53 to Mdm2. I investigated this possibility, but in contrast to my expectations, Fam83F did not reduce the interaction of p53 with Mdm2. Mdm2 is a RING-domain containing E3 ubiquitin ligase that associates with an E2 ubiquitin-conjugating enzyme to facilitate the transfer of the ubiquitin onto p53 (Fang et al., 2000; Honda and Yasuda, 2000). Since Fam83F did not interact with Mdm2, but strongly reduced its ubiquitination, it is possible that Fam83F associates with the E2

enzyme and reduces the association of the E2 enzyme and Mdm2. This could explain why p53 and Mdm2 ubiquitination is reduced by Fam83F without interacting with Mdm2. A further possible explanation how Fam83F could stabilize p53 is related to the shuttling activity of p53 and Mdm2. p53 and Mdm2 shuttle back and forth between the nucleus and the cytoplasm (Liang and Clarke, 2001) and this shuttling activity is important for the degradation of p53 in the cytoplasm. Thus, Fam83F could eventually stabilize p53 by sequestering it in the nucleus. This would then protect p53 from its degradation in the cytoplasm. Therefore, I examined whether Fam83F sequesters p53 in the nucleus. However, cell fractionation showed that Fam83F has no influence on p53 localisation.

## 4.2 Fam83F enhances p53's transcriptional activity

The p53 protein is a transcription factor that regulates the expression of a large number of target genes (Vogelstein et al., 2000). By this p53 can induce a number of different responses ranging from cell death, induction of cell cycle arrest and senescence to DNA repair (Vogelstein et al., 2000; Khoo et al., 2014). In order to investigate whether the increase in p53 abundance caused by Fam83F is also reflected by an increase in p53 activity, I did a reporter assay and I could show that overexpression of *fam83F* enhanced p53 activity strongly in MCF7 cells. One of the major activities of p53 is inducing cell cycle arrest to inhibit the proliferation of cancer cells (Vogelstein et al., 2000; Khoo et al., 2014). I could furthermore show that overexpression of *fam83F* enhanced p53 activity significantly in a colony forming assay. Vice versa, downregulation of endogenous Fam83F in RKO cells resulted in a decrease in *p21* expression that was further validated in a proliferation assay where RKO cells showed enhanced cell proliferation after knocking-down Fam83F.

## 4.3 Fam83F and mutant p53

Fam83F expression was shown to be regulated by miR-143 through decreasing its mRNA stability in cells from oesophageal carcinoma (ESCC; Mao et al., 2016). The role of Fam83F in the control of cell proliferation was investigated in human ESCC cell lines including the cell lines KYSE410 and ECA109 by performing a colony forming assay. The result of this assay showed that a miR-143-resistant mutant form of Fam83F attenuated the anti-proliferative effect of miR-143. p53 is mutated in about 50% of all human cancer (Bykov and Wiman, 2003). ESCCs have several specific molecular alterations and apart from tumour cell aneuploidy, p53 mutations are a hallmark of ESCCs (Reid et al., 2001; Wongsurawat et al., 2006; Souza, 2010). Since in this recent report of Fam83F regulation by miR-143 (Mao et al., 2016) there was no analysis of p53 mutations, it is possible that p53 was mutated in these cells.

Surprisingly, co-immuneprecipitations showed an association between Fam83F and mutant p53 (R273H and R175H). This result opens a window for further research into the role of Fam83F in the regulation of mutant p53. The infulance of Fam83F on mutant p53 might explain the speculation that Fam83F has a dual funcation dependant on the status of p53 in the cell. I could show that Fam83F does not affect the abundance of mutant p53 (R273H and R175H) in the presence of Mdm2, possibly because mutant p53 is already a very stable protein. Fam83F is most likely unable to regulate the abundance of mutant p53, since Fam83F acts by stabilizing wild type p53 in Mdm2 dependant manner and tha mutant forms of p53 are already very stable proteins. This has been shown in previous reports that used the increased stability and thus high abundance of mutant p53 as a diagnostic indicator for stable p53 in human cancers by immunohistochemistry (IHC; Hall and Lane, 1994). Most tumour suppressor proteins are usually downregulated in various tumours. The p53 protein is unique in this regard since high levels of missense mutations of p53 are present in various tumours. These levels of mutant p53 are clearly higher than wild-type p53 in normal unstressed cells (Olivier et al., 2002). Such high levels of p53 furthermore correlated with a particularly aggressive behaviour of the tumours

(Oshiro et al., 1998; Petitjean et al., 2007; Rivlin et al., 2011). In contrast to wild-type p53, mutant p53 does not form a feedback-loop with Mdm2 as mutant p53 does not induce Mdm2 transcription (Midgley et al., 1997). A possible explanation that Fam83 does not stabilize mutant p53 is that mutant p53 escaped the Mdm2 degradation pathway that is required by Fam83F to stabilize the wild type p53.

## 4.4 Fam83F is induced in response to DNA damage

DNA damage efficiently induces a p53 response, in part through the activation of ATM/ATR kinases that result in the phosphorylation of p53 and Mdm2. This phosphorylation can inhibit the interaction between these two proteins, allowing the stabilization of p53 (Banin et al., 1998; Khosravi et al., 1999; Sakaguchi et al., 2000). In addition to the stabilization of p53, DNA damage-induced kinases also plays a role in the activation of p53 as a transcription factor, in part by promoting acetylation within the C-terminus of p53 (Lambert et al., 1998; Sakaguchi et al., 1998; Ou et al., 2005). Since Fam83F induces p53 transcriptional activity through its interaction with the C-terminus of p53, I tested whether Fam83F also induces p53 activity in response to DNA damage. Indeed, Fam83F was induced in response to DNA damage. The expression of *p21* can be directly transactivated by wild-type p53. Knockdown of Fam83F resulted in a reduction of p21 levels when cells were treated with DNA damaging agents. This result suggests that p53 activity is reduced upon Fam83F knockdown in response to DNA damage. Although *p21* expression was reduced after downregulation of Fam83F in RKO cells. I was not able to see a reduction in p53 abundance. This result indicates that p53 accumulates but is functionally impaired by the Fam83F knockdown. Accumulation of p53 and activation are not necessarily directly linked, as shown previously by Gottifred and co-workers. Most interestingly, they could show that despite higher levels of p53 after ionising radiation compared to treatment with hydroxyurea, they were unable to detect an accumulation of p21 or Mdm2 (Gottifredi et al., 2000).

## 4.5 Conclusions

With this work, I can add a new piece to the puzzle of p53 regulation. I could show that Fam83 induces the abundance of wild type p53 in a proteasome-dependent manner by reducing its ubiquitination. Fam83F interacts with the C-terminal domain of p53 that holds the main ubiquitination sites. This interaction via the C-terminus suggests that Fam83F may reduce ubiquitination of p53 by masking the lysine residues in the C-terminal domain of p53. The increase in p53 abundance was furthermore reflected by an increase in p53 activity. Fam83F was upregulated in response to DNA damage and knockdown of Fam83F reduced the expression of *p21* which resulted in increased proliferation.

# 5. Outlook

Fam83F is a relatively uncharacterized protein. Until 2014, there were no publications about the role of Fam83F in the cell. Thus, it was a gene with unknown function. The first observation about Fam83F as a positive regulator of p53 was identified in our laboratory (Zhang et al., 2015).

In 2016, it was published that Fam83F is regulated by miR-143 and that it is upregulated in oesophageal squamous cell carcinoma (ESCC; Mao et al., 2016). Introducing miR-143 into ESCC cells downregulated Fam83F and resulted in inhibition of cell proliferation. Other researchers also working on ESCC found that Fam83F is also regulated by miR-455-3p although they did not show that the regulation of cell proliferation by miR-455-3p is really due to the regulation of Fam83F (Yang et al., 2017). My research and these reports open a new window for research. Since p53 is frequently mutated in human cancer and p53 mutation is a hallmark in ESCCs, it is possible, that p53 was mutant in the ESCC cells that were investigated by Mao and colleagues. During my investigation of the molecular mechanism of p53 regulation by Fam83F, I found that Fam83F also interacts with mutant p53. This raises the possibility that Fam83F might eventually also activate mutant p53 and may act as a tumour suppressor or an oncogene, depending on the status of p53 in the cell.

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# ABBREVIATIONS

| Abbreviation | Full name |
|---|---|
| **A** | Ampere |
| **Aa** | Amino acid |
| **Ac** | Acetylation |
| **APS** | Ammonium persulfate |
| **ATM** | Ataxia-telengiectasia mutated |
| **ATP** | Adenosine triphosphate |
| **ATR** | Ataxia-telengiectasia and Rad3-related |
| **Bcl-2** | B-cell leukemia/lymphoma 2 |
| **Bcl-XL** | B-cell lymphoma extra large |
| **Bid** | BH3-interacting domain |
| **Bp** | Base pairs |
| **BSA** | Bovine serum albumin |
| **$^{o}$C** | Degrees Celsius |
| **$CaCl_2$** | Calcium chloride |
| **CAK** | Cdk (cyclin-dependent kinase)-activating kinase |
| **CBP** | CREB (cAMP-response element binding) – binding protein |
| **CDK** | Cyclin-dependent kinase |
| **cDNA** | complementary DNA |
| **CHIP** | carboxyl terminus of Hsc70-interacting protein |
| **CHX** | Cycloheximide |

## Abbreviations

| | |
|---|---|
| **Co-IP** | Co-immunoprecipitation |
| **CRM-1** | Chromosomal region maintenance-1 |
| **$C_T$** | Cycle threshold |
| **C-terminal** | Carboxy- terminal |
| **d** | Days |
| **DBD** | DNA binding domain |
| **DMEM** | Dulbecco's modified eagle's medium |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic Acid |
| **DNase** | Deoxyribonuclease |
| **dNTPs** | Deoxynucleosides triphosphate |
| **DTT** | Dithiothreitol |
| **DUF** | Domain of unknown function |
| **ECL** | Enhancer of chemioluminescence |
| **EDTA** | Ethylenediamine tetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **e.g.** | Exampli gratia, for example |
| **EGFR** | Epidermal growth factor receptor |
| **et al.** | Et alii, and others |
| **Eto** | Etoposide |
| **Fam83F** | Family with sequence similarity 83 member F |
| **FBS** | Fetal bovine serum |
| **FBXW-7** | F-box and WD-repeat domain containing 7 |

## Abbreviations

| | |
|---|---|
| **f.c** | final concentration |
| **Fig.** | Figure |
| **FLV** | Friend leukemia virus |
| **g** | Gram |
| **g** | Gravity (unit of relative centrifugal force) |
| **GADD45A** | Growth arrest and DNA-damage-inducible protein 45 alpha |
| **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **GoF** | Gain of funcation |
| **GSK-3β** | Glycogen synthase kinase-3 beta DGlycogen synthase kinase- 3 beta |
| **GST** | Glutathione S-transferase |
| **h** | Hour |
| **HAT** | Histone acetyltransferase |
| **HAUSP** | Herpesvirus-associated ubiquitin-specific protease |
| **HCl** | Hydrochloric acid |
| **HDAC** | Histone deacetylase |
| **HECT** | Homologous to the E6-AP Carboxyl Terminus |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **His** | Histidine |
| **HRP** | Horseradish peroxidase |
| **HU** | Hydroxyurea |
| **i.e.** | id est, that is |
| **IF** | Immunofluorescence |
| **IP** | Immunoprecipitation |

## Abbreviations

| | |
|---|---|
| **ITG** | Institute of Toxicology and Genetics |
| **KCl** | Potassium chloride |
| **kDa** | Kilodalton |
| **KRAS** | Kirsten rat sarcoma |
| **L** | Liter |
| **Leu** | Leucine |
| **Lys** | Lysine |
| **M** | Molar |
| **m** | Milli |
| **MAPK** | Mitgen-activated kinase |
| **Mcl-1** | Myeloid leukemia cell differentiation protein-1 |
| **Mdm2** | Murine double minute 2 |
| **Me** | Methylation |
| **MLL1/2** | Mixed lineage leukemia protein-1/2 |
| **MMS** | Methanesulfonate |
| **MOI** | Multiplicity of infection |
| **MOM** | Mitochondria outer membrane |
| **MOZ** | Monocytic leukemia zinc finger protein |
| **mRNA** | Messenger RNA |
| **n** | Nano |
| **NaCl** | Sodium chloride |
| **NES** | Nuclear export signal |
| **Ni-NTA** | $Ni^{2+}$-nitrilotriacetic acid |

## Abbreviations

| | |
|---|---|
| **NLS** | Nuclear localisation signal |
| **NoLS** | Nucleolar localization signal |
| **NP-40** | Nonident P-40 |
| **N-terminal** | Amino- terminal |
| **OD** | Optical density |
| **Oligo** | Oligomerization domain |
| **ON** | Overnight |
| **P** | Phosphorylation |
| **P** | Pico |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PBS-(T)** | Posphate buffer saline-(Tween 20) |
| **PCAF** | P300/CBP associated factor |
| **PCNA** | Proliferating cell nuclear antigen |
| **PCR** | Polymerase chain reaction |
| **pH** | Potential of hydrogen |
| **Phe** | Phenylalanine |
| **PML** | Promyelocytic leukemia protein |
| **PMAIP1** | Phorbol-12-myristate-13-acetate induced protein 1 |
| **PMSF** | Phenylmethanesulphonyl fluoride |
| **PR** | Proline rich domain |
| **PTEN** | Phosphatase and tensin homolog |
| **PTMs** | Posttranslational modifications |
| **PTP** | Permeability transition pore |

## Abbreviations

| | |
|---|---|
| **PUMA** | P53-upregulated modulator of apoptosis |
| **PVDF** | Polyvinylidenedifluoride |
| **qRT-PCR** | quantitative real-time polymerase chain reaction |
| **RE** | Responsive element |
| **REG** | Regulatory domain |
| **RING** | Really Interesting New Gene |
| **RNA** | Ribonucleic acid |
| **RNase A** | Ribonuclease A |
| **Rpm** | Revolutions per minute |
| **RSV LTR** | Rous sarcoma virus long terminal repeat |
| **RT** | Room temperature |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **SD** | Standard deviation |
| **SDS** | Sodium dodecyl sulfate |
| **Sec** | Second |
| **Ser** | Serine |
| **SIRT1** | Sirtuin 1 |
| **Smyd2** | Set and Mynd-domain containing protein 2 |
| **SUMO** | Small ubiquitin-like modifier |
| **SV-40** | Simian virus 40 |
| **SWI/SNF** | SWItch/sucrose Non Fermentable |
| **TA** | Transactivation domain |
| **TAFs** | TATA-binding protein associated factors |

## Abbreviations

| | |
|---|---|
| **TBS** | Tris buffer saline |
| **TEMED** | N,N,N’,N’Tetramethylethylenediamine |
| **TERT** | Telomerase reverse transcriptase |
| **TET** | Tetramerization domain |
| **TFIID** | Transcription factor II D |
| **TPp53** | Tumor protein p53 |
| **Tris** | Trishydroxymethylaminometane |
| **Trp** | Tryptophan |
| **U** | Units |
| **UV** | Ultraviolet |
| **VEGFR-2** | Vascular endothelial growth factor-2 |
| **V** | Volt |
| **WB** | Western blot |
| **w/o** | Without |
| **Wt** | wild-type |