Functions and Regulation of Hdmx and Post-Translational Modifications in Drug Sensitivity and Cancer.

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Table of Contents

Outline of the thesis

Chapter 1 General Introduction

Chapter 2 Functions of MDMX in the Modulation of the p53-Response

Chapter 3 c-Abl phosphorylates Hdmx and regulates its interaction with p53


Chapter 5 Alternative splicing of Hdmx predicts p53 inactivation and bad prognosis

Chapter 6 Function and regulation of SUMOylation in the p53 pathway: a role for RNF4?
Kristiaan Lenos, Kirsten Lodder, Aart G. Jochemsen

Chapter 7 FAU expression is essential for proliferation and affects the drug sensitivity of osteosarcoma cells.
Kristiaan Lenos, Kirsten Lodder, Amina F.A.S. Teunisse, Aart G. Jochemsen

Chapter 8 Summary and General Discussion

CV Nederlandse Samenvatting

Appendices List of Abbreviations
Outline of the thesis

In this thesis the role and regulation of the negative p53 regulator Hdmx (Mdmx/Mdm4) are the main topics. Whereas the role of its close homolog Hdm2 is elaborately studied, the function of Hdmx in tumourigenesis less understood. Post-translational modification of Hdmx is abundant and may play an important role in the regulation of its function. However, relatively little is known about the nature of these modifications. The aim of this thesis was to provide more insight in the putative oncogenic role of Hdmx and to elucidate some of the mechanisms by which Hdmx function is regulated.

The work in this thesis is presented as follows:

Chapter 1 introduces the p53 pathway and it describes the main aspects of the regulation of p53 by Hdm2 and Hdmx.

In Chapter 2 the present knowledge about the regulation of Hdmx in relation to the p53 response is presented.

Chapter 3 describes the effects of c-Abl mediated phosphorylation on tyrosine residues 55 and 99 of Hdmx, which both modulate the interaction with p53.

In chapter 4 the oncogenic functions of Hdmx have been investigated, using an in vitro transformation model of human primary fibroblasts and embryonic retinoblasts.

The function of Hdmx overexpression and alternative splicing in osteosarcoma is described in Chapter 5. A model for a role of Hdmx in early tumours and loss of Hdmx in later stage tumours is proposed, with a key role for the change in ratio between the expression of the alternative Hdmx splice variant, Hdmx-S and full length Hdmx.

Chapter 6 shows that p53 and its regulators Hdm2 and Hdmx are SUMOylated and ubiquitinated and how the SUMO-specific ubiquitin E3 ligase RNF4 might be involved in these processes.

In chapter 7 the function of the FAU gene, encoding the ubiquitin-like protein FUBI and the ribosomal S30 protein, has been studied in osteosarcoma cells.

Chapter 8 is a general discussion and provides a summary of the thesis.
Chapter 1

General Introduction
General Introduction

Chapter 1   General Introduction

Tumour suppressor p53 is regulated by Hdm2 and Hdmx

Cell growth and proliferation are under tight control of the tumour suppressor p53, to prevent uncontrolled cell division or replication of damaged DNA, thereby maintaining the genomic integrity and inhibiting the formation of tumours 1. When cells are stressed or when DNA damage occurs, p53 gets activated, leading to a p53 response which eventually may lead to growth arrest or even apoptosis when damage is unrepairable 2. The importance of this “Guardian of the Genome” function 3 is illustrated by the high frequency (~50%) of p53 mutations in tumours 4,5, whereas in wild-type p53 tumours the p53 tumour suppressor pathway is assumed to be functionally inactivated 6. Furthermore, mice that lack p53 develop almost normally, but are very susceptible to spontaneously formed tumours 7. Obviously, a tight control of the activity of p53 is essential for normal cell growth and cell cycle progression, as well for an appropriate response to damage and stress 8,9. Two of the main p53 regulators are Hdm2 (Mdm2, Mouse double minute 2) and Hdmx (Mdmx/Mdm4, mouse double minute x/4) 10, the importance of which has been demonstrated by the embryonic lethality of Mdm2- and Mdmx-deficient mice, which could both be rescued by p53 loss 11-15. Hdm2 and Hdmx are homologues, showing high similarity in their general structure, and their amino acid sequence is highly similar in the N-terminal p53 binding domain and RING finger domain 16 (Fig. 1).

Figure 1. Protein structure of Hdm2 and Hdmx

Protein structure of Hdm2 and Hdmx, indicated are the functional domains of both proteins. Percentage of similarity between both proteins is shown in between. NLS: nuclear localisation signal; NES: Nuclear Export Signal; Zn: Zinc finger; RING: Really Interesting New Gene; NoLS: Nucleolar Localisation Signal. Adapted from Marine et al. (2007) 108.
Both proteins bind p53 at the N-terminus, thereby inhibiting p53's transcriptional activity\textsuperscript{17,18}. Hdm2 is an E3 ubiquitin ligase for p53, targeting p53 for ubiquitination and proteasomal degradation\textsuperscript{19,20}, which is dependent on the RING and acidic domains\textsuperscript{21,22}. In contrast to Hdm2, Hdmx is unable to fulfil this function by itself, despite its similarity with Hdm2, although dimerization of both proteins stabilizes Hdm2 and enhances the E3 ligase activity towards p53\textsuperscript{23-25}. Furthermore, Hdm2 is not only an E3 ubiquitin ligase for p53, but also ubiquitinates Hdmx and itself, especially after several types of cellular stresses, like DNA damage\textsuperscript{26-28}. Other ubiquitin E3 ligases for p53 have been described\textsuperscript{29,30}, however, it is generally believed that Hdm2 is the main E3 ligase in the regulation of p53 stability.

Regulation of Hdmx levels

Whereas under normal growth conditions the Hdmx protein is stable, after DNA damage its levels rapidly decrease, due to Hdm2-mediated degradation\textsuperscript{31}. For a long time, Hdmx expression was considered to be regulated only at the protein level. However, the group of Jiandong Chen showed that the Hdmx promoter is responsive to mitogenic stimulation via the Ets-transcription factor family members\textsuperscript{32}. Recently it was discovered that, similar to Hdm2, the Hdmx gene contains a p53 responsive promoter, which is activated upon p53 activation, thereby creating a negative feed-back loop to attenuate the p53 response\textsuperscript{20,33-35}. The mRNA synthesized from the p53-responsive P2-promoters of both Hdm2 and Hdmx are much more efficiently translated than the mRNAs transcribed from the constitutive P1-promotors\textsuperscript{34,36-38}.

Several alternative splice variants of Hdm2 have been described\textsuperscript{39} and up to now six alternative spliced forms of Hdmx have been detected\textsuperscript{40,41}. At least one of these Hdmx splice variants, Hdmx-S, might be implicated in tumourigenesis, as its mRNA expression was found to be upregulated in a number of tumours and the expression of Hdmx-S correlates with worse patient survival\textsuperscript{42-45}.

Hdmx can act as an oncogene

Whereas a lack of Hdm2 or Hdmx in normal cells containing wild-type p53 in most cases is lethal for cells, overexpression or amplification of these genes can lead to tumourigenesis. By overexpression of one of these proteins, p53 activity can be sufficiently repressed or inactivated to overcome oncogenic stress-induced growth arrest or senescence. Up to 10% of all tumours show overexpression of Hdm2, usually in the presence of wild-type p53, revealing Hdm2 as an oncogene\textsuperscript{46}. Similarly, Hdmx can act as an oncogene; increased Hdmx mRNA expression was found in 20% of tumours in a study of common tumour types\textsuperscript{47} and a subset of gliomas showed amplification of the Hdmx gene\textsuperscript{43}. In a large number of
wild-type p53 tumour cell lines upregulated or aberrant Hdmx protein expression was found. Furthermore, retinoblastomas, which nearly all retain wild-type p53, show amplification of the hdmx gene in a particularly high proportion of tumours, thereby overcoming the p53-mediated apoptosis after Rb (Retinoblastoma protein 1) loss during tumour progression. Moreover, around 90% of Ewing Sarcomas show wild-type p53 and a high number of these tumours show Hdmx overexpression.

Danovi et al. (2004) were the first to demonstrate an oncogenic activity of murine Mdmx, by showing that overexpression of Mdmx combined with HRasV12 in mouse embryonic fibroblasts resulted in immortalization and neoplastic transformation. Recently, two transgenic mouse models have been described that widely overexpress the Mdmx gene. Interestingly, only in one model the development of spontaneous tumours was found. The oncogenic activity of Hdmx in human cells had not yet directly been investigated. We show in this thesis, Chapter 4, that increased Hdmx levels can replace the loss of p53 in the transformation of human fibroblasts and embryonic retinoblasts.

A putative role for Hdmx as a driving factor for tumourigenesis may also be found in the bone malignancy osteosarcoma. Since only around 20% of high-grade osteosarcoma has p53 mutations and Hdm2 was found to be amplified in only 10-35% of osteosarcoma, Hdmx overexpression might be an additional way to overcome p53 inhibition. In this thesis, Chapter 5, we show that Hdmx overexpression may be a mechanism to inhibit p53 in early stage osteosarcoma, whereas in later stages, p53 is usually inactivated in other ways. We found that Hdmx expression in these late stage tumours with inactivated p53 is reduced, whereas the alternative Hdmx splice variant Hdmx-S is strongly upregulated. This change in the ratio between these splice-variants turned out to be a stronger prognostic marker than p53 mutational status only.

Treatment of tumours that retain wild-type p53 with compounds that reactivate p53 is a promising therapy. By relieving the inhibitory pressure of Hdm2 or Hdmx on p53, an effective anti-cancer therapy might be developed. Small molecule inhibitors such as Nutlin-3, which interferes with the binding of Hdm2 and p53, have already been used in many studies and are being tested in clinical trials. More recently, also specific inhibitors of Hdmx have been developed. An interesting development is the use of Nutlin-3 and similar inhibitors in so-called cyclotherapy, in which the activation of p53 serves as a chemoprotectant in normal cells, whereas tumour cells will become even more sensitive to the chemotherapeutic treatment. This approach will also function in the treatment of mutant p53 tumours, since these cells, in contrast to normal cells, will not arrest in G1 and
G2 after p53 activation and, therefore, specifically the tumour cells will be targeted by the following chemotherapy.

Post-translational modifications in the p53 pathway: Cross-talk on the lysines.
The regulation of p53 levels and activity is mainly at the protein level; changes in protein stability and decreased functional inhibition allow p53 and p53 target gene levels to rise very quickly upon stress signals. Post-translational modifications play a critical role in the p53 regulation. The most obvious modification is the conjugation of ubiquitin to p53 by Hdm2, resulting in altered subcellular localization when p53 is mono-ubiquitinated, or proteasomal degradation as a result of poly-ubiquitination\textsuperscript{61-63}. Several other ubiquitin-like modifications have been reported to be involved in p53 regulation, such as SUMOylation and NEDDylation, of which the conjugation of the small ubiquitin related modifiers, SUMO’s, is the best studied. The conjugation of SUMO to p53 by Hdm2 or PIAS family members has been reported to inhibit p53 transcriptional activity, although others found an activation of p53 activity upon SUMOylation\textsuperscript{64-66}. Hdm2 and Hdmx are also SUMOylated on two or more lysines, which have been implicated in the function of Hdm2\textsuperscript{67-70}. Interestingly, the conjugation of SUMO and ubiquitin cross-talk in several ways\textsuperscript{71}. SUMOylation of proteins has been reported to interfere with ubiquitination. On the one hand by shielding the target lysine for ubiquitination, or by SUMOylation of components of the ubiquitination system\textsuperscript{72}. Moreover, the recent discovery of SUMO Targeted Ubiquitin Ligases (STUbLs), such as the human Ring Finger Protein 4, RNF4, adds a new twist to the interplay between ubiquitination and SUMOylation\textsuperscript{73,74}. This type of E3 ubiquitin ligases specifically ubiquitinates (poly-)SUMOylated targets, through recognition by a SUMO Interacting Motif (SIM), of which the ubiquitination of poly-SUMOylated PML (promyelocytic leukemia protein) by RNF4 was the first example\textsuperscript{75-77}.

Other post-translational modifications on lysines such as acetylation and methylation have also been implicated in the regulation of the activity and interactions of p53, Hdm2 and Hdmx. Acetylation of p53 C-terminal lysines is upregulated after DNA damage and has been suggested to have an activating effect, by shielding these residues for ubiquitination or other inhibiting modifications. NEDDylation, methylation and SUMOylation can occur at unique lysines in p53, but may also compete with ubiquitination and acetylation for the same residues, resulting in a fine-tuning mechanism of p53 activity and stability\textsuperscript{78-82}.

Post-translational Modifications in the p53 pathway: Phosphorylation
For the activation of p53 upon DNA damage, certain phosphorylation events on p53, Hdm2 and Hdmx are crucial. Upon DNA damage, kinases such as ATM, ATR, DNA-PK and others are activated and start a phosphorylation signaling cascade. Numerous
phosphorylations on p53 are known, including a cluster of N-terminal residues of which S15, T18 and S20 are the most well studied. Phosphorylation on S15 appears to increase the interaction between p53 and the transcriptional co-factors p300/CBP, while phosphorylation of T18 and S20 results in decreased affinity for Hdm2\textsuperscript{83,84}. Phosphorylation of S46 is associated with the induction of apoptosis\textsuperscript{84,85}. Of the C-terminal phosphorylations, phosphorylation of S392 has been reported to stabilize p53 tetramerization and enhance specific DNA binding, while S351 phosphorylation was suggested to activate p53 transcription activity\textsuperscript{80}. Next to the activating phospho-modifications on p53 itself, the negative regulators of p53, Hdm2 and Hdmx, are inactivated due to phosphorylation upon DNA damage. The phosphorylation of sites near the RING domain of Hdm2 prevents the oligomerization of Hdm2, thereby attenuating p53 ubiquitination and degradation\textsuperscript{86}. Phosphorylation of Hdmx C-terminal serines S342, S367 and S403 promotes its degradation by Hdm2\textsuperscript{87-89}. Similarly, the phosphorylation of Hdm2-S395 by ATM induces auto-ubiquitination of Hdm2\textsuperscript{90}. Furthermore, c-Abl-mediated phosphorylation of Hdm2 on the adjacent Y394 inhibits the nuclear export and degradation of p53\textsuperscript{91-93}. Additionally, phosphorylation of S17 on Hdm2 by DNA-PK has been reported to disrupt the Hdm2-p53 binding\textsuperscript{94} and we have shown that tyrosine-99 phosphorylation of Hdmx by c-Abl exerts a similar function (this thesis, Chapter 3,\textsuperscript{95})

Phosphorylations on p53, Hdm2 and Hdmx affect the affinity of the Ubiquitin Specific Protease USP7 (HAUSP) for these proteins. Under normal growth conditions, USP7, a de-ubiquitylase (DUB), removes ubiquitin from Hdm2 and Hdmx, which is essential for maintaining levels that are sufficient to inhibit p53 activity. However, upon DNA damage the interaction of USP7 with Hdm2 and Hdmx is attenuated, leading to their destabilization and inability to inhibit p53, while p53 still is a USP7 target\textsuperscript{96,97}.

Whereas the above described phosphorylations on Hdm2 and Hdmx are induced by DNA damage and prevent the inhibition and degradation of p53, phosphorylations by other (pro-survival) kinases such as Akt, CK1-alpha or GSK3 are involved in the stabilization and functional regulation of Hdm2 and Hdmx and have been reported to inhibit p53 function\textsuperscript{78,98,99}.

The \textit{FAU} gene encodes the S30 ribosomal gene and the ubiquitin-like FUBI and functions in apoptosis.

Another Ubi-Like protein, probably the least studied, is FUBI. FUBI is translated from the \textit{FAU} (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-Associated Ubiquitously expressed gene) mRNA, as a fusion product with the ribosomal S30. Like ubiquitin-precursor proteins, cleavage occurs after a Gly-Gly motif, generating the mature FUBI protein. The \textit{FAU} gene was originally identified as a pro-apoptotic gene\textsuperscript{100} and its
downregulation is associated with tumourigenicity and drug resistance\textsuperscript{101-104}. Moreover, a number of reports demonstrate the binding between the pro-apoptotic p53 target Bcl-G and FUBI, which seems to be essential for the induction of apoptosis by FAU\textsuperscript{102,103,105,106}. On the other hand, in the osteosarcoma cell line HOS, FAU and FUBI overexpression was found to have transforming capabilities, whereas FAU and S30 overexpression conferred arsenite resistance\textsuperscript{107}. In this thesis, Chapter 7, we investigated the function of the \textit{FAU} gene in osteosarcoma and found, in contrast to what was published before, that \textit{FAU} expression is essential for normal proliferation. Furthermore, we demonstrated that \textit{FAU} expression affects the sensitivity to several drugs.
General Introduction

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General Introduction


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General Introduction

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Chapter 2

Functions of MDMX in the Modulation of the p53 Response

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Chapter 2

Abstract
The MDM family proteins MDM2 and MDMX are two critical regulators of the p53 tumor suppressor protein. Expression of both proteins is necessary for allowing the embryonal development by keeping the activity of p53 in check. Upon stresses that need to activate p53 to perform its function as guardian of the genome, p53 has to be liberated from these two inhibitors. In this review we will discuss the various mechanisms by which MDMX protein levels are downregulated upon various types of stress, including post-translational modifications of the MDMX protein and the regulation of mdmx mRNA expression, including alternative splicing. In addition, the putative function(s) of the described MDMX splice variants, particularly in tumor development, will be discussed. Lastly, in contrast to common believe, we have recently shown the existence of a p53-MDMX feedback loop, which is important for dampening the p53-response at later phases after genotoxic stress.
Functions of MDMX in the Modulation of the p53 Response

Introduction

The tumor suppressor protein p53 is maintaining genome integrity following stress signals by either inducing a cell cycle arrest, allowing a complete check-up of the genome before resuming DNA replication, inducing senescence, or by stimulating apoptosis when the cell is beyond rescue [1]. In addition, p53 has been shown to directly affect DNA repair by modulating expression of number of DNA repair genes [2]. During tumor development, inhibition of angiogenesis is part of the tumor suppressor function of p53 [3]. In recent years, it has been shown that p53 also affects the metabolic switch which occurs in many tumors, in which energy is obtained by glycolysis rather than via oxidative phosphorylation [4]. p53 is activated by a plethora of stimuli, including DNA damage, hypoxia and oncogene activation, the final outcome of the activation being very dependent on the strength and type of the stimulus and the type of targeted cell.

Whatever stimulus used to activate p53, it has become clear that for full, but controlled p53 activation, a tight control of activity and levels of the main p53 inhibitors, MDM2 and MDMX is necessary [8] [9]. Various mouse models provide the best evidence regarding the importance of MDM2 and MDMX in controlling p53 activity under normal conditions. Loss of either MDM2 or MDMX expression leads to embryonal lethality, which is rescued by simultaneous loss of p53. Tissue-specific deletion of either the \textit{mdm2} gene or the \textit{mdmx} gene showed differences between cell types for their dependency on MDM2 and MDMX to keep p53 in check. These mouse models have recently been discussed in two excellent reviews [9] [10].

The MDMX and MDM2 proteins have a similar structural organization (Figure 1). MDM2 and MDMX bind via their N-terminal hydrophobic region to a short alpha-helical stretch within the p53 N-terminus [11]; this N-terminal region is best conserved between the two proteins. Other conserved structures include the C-terminal RING finger domain, a Zn-finger and a central acidic region. The RING finger domains of MDMX and MDM2 are involved in their homo- and hetero-multimerization [12]. MDM2 has convincingly been shown to form both homo-oligomers and hetero-oligomers with MDMX. In contrast, no significant homo-oligomerization of MDMX could be found. This deficiency could have strong implications for the function of MDMX (see below).

MDM2 is an E3 ubiquitin ligase that targets p53 for ubiquitination and degradation. Essential for p53 ubiquitination are both the MDM2 C-terminal region including the RING finger and the acidic domain. Corresponding regions of MDMX cannot fulfill this function [13-14]. MDM2-mediated mono-ubiquitination of p53 leads to nuclear export, thereby inhibiting the p53’s function as a transcription activator.
Moreover, it has become evident that Mdm2-dependent mono-ubiquitination of p53 promotes the localization of p53 to the mitochondria, where stress induced HAUSP-p53 complexes result in non-ubiquitinated, apoptotically active p53 [15]. Low levels of Mdm2 favor mono-ubiquitination of p53, leading to this mitochondrial transcription-independent induction of apoptosis upon stress. In contrast, high levels of Mdm2 and/or in combination with p300/CBP, promote poly-ubiquitination, which results in proteasome-dependent degradation of p53 [16]; [17]. As mentioned, MDMX has no detectable ubiquitin-ligase itself, but has been proposed to inhibit p53 in a dual way. First, MDMX binds tightly to the N-terminal transcription activation domain of p53, in that way inhibiting its function as transcription activator [18]. Binding of MDMX to this part of p53 prevents the interaction of p300, which results in reduced acetylation of p53 which is involved in activation of p53 [19-20]. Interestingly, p300 acetylates several lysines at the C-terminal region of p53 that are also targeted by Mdm2-mediated ubiquitination [17]. This might implicate that MDMX indirectly stimulates the MDM2-mediated ubiquitination by reducing the acetylation of those lysines. It should be noted that the importance of modifications of the C-terminal lysines for correct regulation of p53 stability and activity in vivo appears to be limited. Krummel et al. [21] showed that a knock-in mutant mouse in which the C-terminal lysines were replaced by arginines developed normally. Several assays showed no difference in regulation of wild-type p53 and p53-7KR. Only a minor increased activation of p53 in the thymus upon ionizing radiation was found, and, interestingly, p53-7KR MEFs could not...
Functions of MDMX in the Modulation of the p53 Response

escape from senescence, in contrast to normal MEFs, indicating an increased activity of p53-7KR. The difficulty with interpretation of the results is that one cannot distinguish between the functions of the various possible modifications on the lysines (ubiquitination, sumoylation, neddylation, methylation, acetylation). It could well be that a certain modification on one lysine counteracts the effect of a different modification on a nearby lysine. In Mdmx-deficient MEFs we have seen increased acetylation of the one analyzed lysine in p53, K379. A full proteomic/mass spec approach would be interesting to analyze the ratio of various lysine modifications at the p53 C-terminus, and the effect of different MDMX expression levels on that ratio.

What has become quite clear is that the ratio between the levels of MDM2/MDMX strongly determines the outcome on p53 stability. A relative high level of MDMX might compete p53 away from MDM2, leading to p53 stabilization [22]. In addition, as mentioned above, MDM2 and MDMX interact via their respective C-terminal RING finger domains, and it has been proposed that under non-stress conditions the MDM2/MDMX heterodimer functions as a more active ubiquitin ligase via providing an extended interaction motif with the E2 protein [23-25]. One might argue that only a dimer/oligomer of MDM2/MDMX RING fingers can bind the E2; since MDMX cannot homo-dimerize, MDMX on its own is defective as a ubiquitin ligase. This might certainly be true, but is not the whole explanation since it has been shown that just replacing the MDMX RING with an MDM2 RING cannot rescue the ubiquitin-ligase activity of MDMX [13-14].

In this review we will focus on the regulation of MDMX expression upon various forms of stress. In most cases, a downregulation of MDMX protein is observed, which is needed for full p53 activation. On the other hand, under certain lethal conditions, a positive role for MDMX in p53-induced apoptosis has been found. These various and apparently contradictory results will be discussed. This includes the recently discovered p53-MDMX feedback loop, which affects the outcome of genotoxic insults in cells.

MDMX regulation upon genotoxic stress

*Inactivation of MDMX at the protein level*

Interestingly, initial reports indicated that MDMX levels were not affected by DNA damage or differentiation or during cell growth [18, 26]. These wrong conclusions were caused by the absence of high-affinity antibodies and by our limited insight into the ways MDMX mRNA levels were regulated. The first to notice that DNA damage influences MDMX were Jiandong Chen and co-workers. They observed that DNA damage induces the nuclear translocation of exogenously expressed MDMX, but an effect on MDMX protein levels
was not discovered [27]. Although it had been reported that MDMX could be degraded by a proteasomedependent mechanism, which is enhanced by p53- and Adriamycin-induced caspase- dependent cleavage of MDMX [28], only a year later it was recognized that MDMX is a target of the ubiquitin-ligase activity of MDM2, and that endogenous MDMX protein is degraded upon DNA damage, in an MDM2-dependent fashion [29-31]. Importantly, overexpression of MDMX prevented p53 stabilization and activation [31]. Notably, in these studies no effect on the total amount of MDMX mRNA by ionizing radiation (10 Gy) was observed. Others and we subsequently showed that the MDM2-mediated degradation of MDMX is strongly stimulated upon ATM-dependent phosphorylations on three serine residues, S342, S367 and S403 [32-34]. These phosphorylations have multiple effects, including the temporal nuclear accumulation of MDMX in a 14-3-3 dependent manner [34-36].

Increased association of MDM2 with MDMX upon phosphorylation of the latter has been suggested [32] as an explanation for the increased ubiquitination and degradation of MDMX. Alternatively, we have shown that upon ATM-dependent MDMX phosphorylation the interaction with the deubiquitinase HAUSP is strongly decreased, and that HAUSP interaction/expression is needed to maintain endogenous MDMX levels [35, 37]. Whether the observed increased interaction with 14-3-3 proteins and the decreased interaction with HAUSP are causally related has not been proven. Interestingly, recently the interaction motifs for HAUSP on human MDMX have been mapped. It was found that both an N-terminal motif (8AQCS11) and more C-terminal motif (398AHSS401) in MDMX can interact with HAUSP [38]. The latter is very close to the S403, which is directly phosphorylated by ATM. Although not tested for this particular motif, it was found that phosphorylation of a serine residue two positions C-terminal of the core motif could strongly inhibit the interaction with HAUSP as assayed by peptide arrays. So, possibly both the direct phosphorylation event on S403, and the interaction of 14-3-3 around phosphorylated S342 and S367, are involved in decreasing the HAUSP/MDMX interaction.

Although one of the HAUSP interaction motifs in MDM2 is also close to the phosphorylation targets Y394 and S395, these phosphorylation events do not appear to affect the interaction with HAUSP [38]. Even though MDM2 lacks classic consensus sites for interaction, 14-3-3 proteins have also been shown to bind MDM2. Two sites have been mapped; one within the middle domain of MDM2, dependent on phosphorylation by Pim1-kinase on S166 and S186 [39]. However, this event appears to stabilize MDM2. On the other hand, 14-3-3σ was shown to interact with the RING finger domain of MDM2, in a phosphorylation-dependent manner, and this interaction enhanced MDM2 ubiquitination and degradation [40].
Functions of MDMX in the Modulation of the p53 Response

Unfortunately, the involved phosphorylation events (target sites, signaling cascade) have not been investigated, but the 14-3-3σ interaction domain was found rather far downstream of S395, which is needed for the temporarily destabilization of MDM2 upon ionizing radiation [41], so a causal relation is not obvious. In addition, whether the observed effects on MDM2 stability are dependent on this specific isoform of 14-3-3 has not been extensively investigated.

The above reported regulation of MDMX protein levels via ATM/Chk2 phosphorylations and 14-3-3/HAUSP interactions were all upon treatment of cells with IR or IR-mimetic NCS (neocarzinostatin). Surprisingly, UV-C induced DNA damage also increases phosphorylation of MDMX/Ser367 in a Chk1-dependent manner, but in this case the increased interaction with 14-3-3 results in cytoplasmic sequestering of MDMX [42]. This manner of MDMX inactivation also turned out to be important for a full p53-stabilization upon DNA damage. Unfortunately, the authors did not investigate total levels of endogenous MDMX after longer time-points after UV-C irradiation; it is not excluded that the observed change in localization, seen three hours after UV-C, is a first step in the inactivation of the MDMX protein, followed by a total disappearance of full length MDMX protein either via proteasomal degradation or via alternative splicing of the MDMX pre-mRNA (see below). The physiological relevance of the UV-C mediated responses is questionable, since humans are not exposed significantly to UV-C. The use of UV-A or UV-B irradiation would have been better in that respect.

A recent elegant study with the use of a mouse knock-in model has shown the importance of the S342, S367 and S403 MDMX phosphorylations, not only for a DNA damage response, but also in the regulation of tumor suppression by p53 [43]. As could be extrapolated from the cell culture studies, thymocytes or MEFs derived from the 3SA mutant mice show decreased degradation of MDMX upon ionizing radiation or neocarzinostatin treatment, and a concomitant reduced p53 stabilization and activation. More striking is the observation that crossing the 3SA mutant mice with Eμ-Myc knock-in transgenic mice strongly decreased the latency period for tumor formation. In the developing tumors, no selection for p53 mutation was found in the 3SA mice, indicating that the expression of the mutant, degradation resistant MDMX is sufficient to inhibit p53's tumor suppressor function. Although the involvement of other mechanisms cannot be excluded, it was found that the overexpression of the Myc transgene did result in activation of the double-strand DNA break DNA damage response, which could explain the observation that prevention of DNA damage-associated phosphorylations of MDMX impact on the tumor development [43]. It should be noted that it is not excluded that other,
yet undefined, kinases are involved in the phosphorylation of MDMX which can be activated by Myc overexpression.

In addition to the serine phosphorylations mentioned above, also tyrosine phosphorylation of MDMX upon imposing double-strand DNA breaks has been reported [44]. Tyrosine 55 and tyrosine 99 were identified as targets of the c-Abl tyrosine kinase. Phosphorylations on these sites increased upon DNA damage. Interestingly, Y99 phosphorylation was found to inhibit the p53/MDMX interaction, which was supported by structural modeling. Function of Y55 phosphorylation is not yet known, but appeared to be dependent on Y99 phosphorylation and the authors suggest a possible role for Y55 phosphorylation in the recovery phase of p53 activation. These results indicate that double-strand DNA breaks not only targets MDMX to activate p53 via the ATM/Chk2 pathway to reduce MDMX levels, but also via a very quick pathway, c-Abl mediated phosphorylation that inhibits the MDMX/p53 interaction.

**Inactivation of MDMX at the post-transcriptional mRNA level**

Extensive alternative mRNA splicing of *mdm2* has been observed, especially in tumor cells [45]. In addition, existence of some splicing variants of *mdmx* has also been reported, mainly in tumors/tumor-derived cell lines (Reviewed recently by [46]). The so far identified *mdmx* mRNA variants obtained by alternative splicing are depicted in figure 2. Lozano and colleagues investigated whether genotoxic stresses would also affect the splicing pattern of *mdm2* and *mdmx* [47]. It was observed that *mdm2* showed alternative splicing upon treatment with rather high doses of UV-C or cisplatin, but IR did not affect the splicing pattern. Similarly, *mdmx* alternative splicing was observed upon 30 or 50 J/m² UV-C, but not with 10 J/m². Unfortunately, the effect of cisplatin treatment on *mdmx* mRNA splicing as not tested in this study. What is missing from these studies is a clear quantification of the levels of the alternative spliced mRNAs compared to the normal spliced mRNA. Even better would have been to investigate whether the putatively produced alternative protein products are indeed synthesized endogenously. For MDMX that might be difficult, because only few high quality antibodies are available, but for MDM2 it could have been performed. Possible functions of the protein products of splicing variants have been investigated almost only by strong overexpression, which might not reflect the physiologically relevant situation. Therefore, no conclusions can be drawn regarding a physiological function of the alternative splicing after these high levels of DNA damage.

The authors note that they also detect low levels of another *mdmx* splicing variant, *mdmx*-S [47]. This splicing variant, a result of exon 6 skipping, was first described by Rallapalli and colleagues [48]. The alternative splicing was mainly found in some cancer cell lines, and could be increased in non-transformed cells upon serum-stimulation. The *mdmx*-S mRNA
encodes essentially only the p53 binding domain, and a number of unique C-terminal amino acids (Figure 2). In overexpression studies the MDMX-S product was found to be a stronger inhibitor of p53 activity, most likely because this short protein localized into the nucleus much more efficiently than full length MDMX [49].

Figure 2. Schematic representation of the MDMX protein, the mdmx mRNA and the reported mdmx splicing variants.

Importantly, in recent experiments we found that after exposure of normal human fibroblasts or MCF-7 cells to relatively low levels of UV-C (10 J/m²), splicing of mdmx mRNA is changed, but that preferentially the mdmx-S variant is expressed (Figure 3). This is a relative quick event in the human skin fibroblasts, and occurs somewhat slower in the MCF-7 cells. Under these conditions, the X-Alt1 and X-Alt2 splice variants as described by Chandler et al. (2006) could not be detected. In addition, we found that cisplatin (30 μM) strongly induced the switch in ratio between full-length mdmx- and mdmx-S mRNAs in the breast carcinoma MPE600 cell line (Figure 3). Similar results were obtained in other tumor cell lines, such as U2OS, upon treatment with comparable concentrations cisplatin or with 20 μM doxorubicin (data not shown).

At the moment it is not known via which mechanism alternative splicing of mdmx is induced upon genotoxic stress. From literature a few mechanisms can be proposed. First, it has been shown that upon stress the subcellular localization of proteins involved in splice site selection is affected, like the hSlu7 protein (3’ site selection) and the hnRNP A1 proteins (5’ site selection). Treatment of cells with osmotic shock or UV-C leads to
cytoplasmic localization of hnRNP A1, which affects the choice of 5' splice site [50]. Since with \textit{mdmx} the change is the choice of the 3' splice site, involvement of hSLu7 is more likely [51]. However, in that publication it is mentioned that cisplatin treatment does not affect hSLu7 subcellular localization, so regulation of hSLu7 is most likely not the single mechanism by which the splicing of \textit{mdmx} mRNA is altered. More recently it was reported that alternative splicing upon UV-C treatment is co-regulated with the inactivation of RNA polymerase II by inducing the hypophosphorylation of the CTD region [52]. Although such a mechanism can not be excluded at the moment in the alternative splicing of \textit{mdmx} mRNA, changes in subcellular localization of certain factors involved in splicing are likely to play a role in the alternative splicing of \textit{mdmx} mRNA. We have found that the treatment of several types of cells with Leptomycin B, which blocks nuclear export of proteins containing a Rev-like nuclear export signal, strongly increases alternative splicing of \textit{mdmx}, leading to increased levels of \textit{mdmx-S} mRNA and reduced \textit{mdmx} full length mRNA (Figure 3), accompanied with a reduced MDMX protein level (not shown).

Even so, it is unclear what the biological importance of this alternative splicing is. It can be a means to reduce the amount of full length MDMX protein (which indeed is found after sublethal doses of UV-C [31, 53]), needed for full activation of p53. In this case, alternative splicing would lead to a loss-of-function. On the other side, it could be a mechanism to produce an MDMX protein form that is resistant to MDM2-mediated degradation, which could have a function in attenuating the p53 response at later time-points after the damage. In that case, it would be a gain-of-function. Putative biological functions of protein products of the \textit{mdmx} splicing variants will be discussed later.

\textbf{Figure 3: The ratio between \textit{mdmx-S} and \textit{mdmx-fl} mRNA is increased by various types of DNA damage and by inhibition of nuclear export.} RT-PCR analysis of RNAs extracted from various cell lines, treated for the indicated time periods with a variety of agents. The \textit{mdmx}-specific PCR primers are located in exon 3 and exon 8. The doses genotoxic/drug treatments used: UV-C: 15 J/m²; cisplatin: 30 μM; LMB (Leptomycin B): 10 nM; Eto (Etoposide): 20 μM; Nutlin-3: 10 μM.
Markey and Berberich [54] have recently uncovered another mechanism leading to reduction of MDMX protein levels upon genotoxic agents. They found that exposing cells to doxorubicin (0.5 μg/ml; which is ~ 0.85 μM) leads to a strong reduction in the total \textit{mdmx} mRNA levels; this effect was found to be fully p53-independent. Similarly, cisplatin reduces \textit{mdmx} mRNA levels in doses varying from 25 to 100 μM, on various cell lines, independent of their p53 status. In addition, the authors show that the decrease of MDMX protein levels after doxorubicin treatment could not efficiently be rescued by proteasome-inhibition or knock-down of p53 and MDM2, when measured 24hrs after start of treatment. It would have been nice if the authors would have shown a time-course of doxorubicin-treatment and analyzed both MDMX protein and mRNA. It could well be that initially degradation of the MDMX protein occurs upon the activation of the DNA damage signaling cascade, and that the reduced mRNA level is a later effect. These authors also investigated the alternative splicing of \textit{mdmx} mRNA upon doxorubicin treatment. The remarkable observation here is that for this experiment they use a 100 μM concentration of doxorubicin, which is more than 100-fold higher than used in their initial experiment, so it cannot be compared to the earlier experiment. It could be, but that is not commented upon, that at the lower concentration of doxorubicin no significant alternative splicing can be found. Anyway, the authors do find some increase in \textit{X-Alt2} levels, but actually a small decrease in \textit{X-Alt1}. Again, total levels of \textit{mdmx} mRNA strongly decrease, indicating that \textit{X-Alt2} only represents a very small fraction of the total \textit{mdmx} mRNA. Interestingly, the decrease in \textit{mdmx} mRNA levels is caused by decreased stability of the \textit{mdmx} mRNA. The authors suggest a putative involvement of microRNA miR-34a in this regulation of \textit{mdmx} mRNA stability. This is interesting in the view of the earlier observation that transcription of the miR34 family members is upregulated upon p53 activation, which has been shown to be important for a full p53 response upon DNA damage [55-56]. However, the regulation of miR-34a by p53 does not explain the p53 independency of the \textit{mdmx} mRNA destabilization upon DNA damage. Unfortunately, it was not addressed in this study whether the decrease in \textit{mdmx} mRNA after doxorubicin or cisplatin treatment is important for the biological outcome of the treatment. So, the physiological importance of the observed \textit{mdmx} mRNA reduction still has to be investigated.

\textit{A pro-apoptotic function of MDMX upon lethal doses of stress}

In all the experiments presented above, the idea was that a decrease in MDMX expression is needed to get a full-blown p53-response upon DNA damage. And indeed, in most cases a decrease in MDMX levels is observed and some experiments have shown that prevention of that MDMX degradation is inhibiting the p53 response [31, 35]. However, these
experiments were all done with a sub-lethal dose of IR or treatment with IR-mimetic agents like NCS.

The group of Fabiola Moretti has obtained results that appear to be in conflict with what has been discussed above. In an initial report it was presented that in an (inducible) overexpression system high levels of MDMX actually stimulate doxorubicin (adriamycin)-induced apoptosis [57]. This correlated with an increased induction of Bax transcription, while p21WAF1 was induced similarly in control- and MDMX-overexpressing cells, suggesting that high MDMX expression facilitated p53-mediated induction of pro-apoptotic genes specifically. These experiments were all performed with the use of non-transformed mouse or human cells, with doxorubicin as the genotoxic agent. The main drawback of this study is that a pro-apoptotic function of endogenous levels of MDMX protein was not investigated. Furthermore, it could be that MDMX has a different function in untransformed versus tumor cells upon adriamycin-treatment. In this respect it is of interest to note that in the colorectal tumor cell line HCT116 an active p53 seems to inhibit adriamycin-induced apoptosis [58], so a pro- or anti-apoptotic function of MDMX could very well be strongly cell type specific.

In a follow-up study the authors made the very intriguing observation that a fraction of MDMX is localized to the mitochondria, and that upon lethal stress MDMX could function as an anchor for S46-phosphorylated p53, leading to transcription-independent apoptosis [53]. Unfortunately, the genotoxic agents used were now UV-C and cisplatin, which makes comparison with the earlier publication difficult. Strikingly, it was found that low levels of UV-C irradiation did reduce MDMX protein levels in MCF-7 cells, but that high, lethal doses (40 J/m²) of UV-C did not or much less. At these high doses, reduced levels of MDM2 were found, fitting with earlier reports that lethal doses of genotoxic agents can inhibit *mdm2* transcription [59-60], partly by activation of HIPK2 [61, 62]. The results also lead to two tentative conclusions. First, that the observed alternative splicing of *mdmx* mRNA after high UV-C doses in MCF-7 cells [47] would not lead to a reduction in full length MDMX protein. Second, that under these conditions the earlier reported downregulation of *mdmx* mRNA levels upon genotoxic stress [54], admittedly a different type, is not occurring. Most strikingly in this study is the observation that reducing MDMX levels by siRNA did decrease the induction of apoptosis upon UV-C irradiation. A similar result was obtained upon treatment of control- and MDMX-knockdown MCF-7 cells with α-amanitin, which blocks polymerase II transcription but is reported to induce a p53-dependent apoptosis strictly by stimulating translocation of p53 to the mitochondria. Similarly, reducing MDMX levels in A2780 cells partly prevents cisplatin-induced apoptosis (20 μM cisplatin). This concentration of cisplatin did not induce significant
alternative splicing of \textit{mdm2} mRNA in MCF-7 or U2OS cells [47]. The effects on \textit{mdmx} mRNA were not investigated in this study, but a similar dose of cisplatin (25 \(\mu \text{M}\)) caused a small upregulation of \textit{mdmx} mRNA in MCF-7, but already a downregulation in some other cell lines [54]. We found recently in a few osteosarcoma cell lines that 20 \(\mu \text{M}\) cisplatin reduces the total levels of \textit{mdmx} mRNA, and increases the \textit{mdmx-S: mdmx-FL} ratio, independent of the p53 status (Lenos et al., manuscript in preparation).

It is difficult to reconcile the data from Moretti and colleagues with the earlier presented data. However, there are a number of points to keep in mind. The data obtained with ionizing radiation or IR-mimetic usually did not induce a high level of apoptosis, but mainly a cell cycle arrest. When UV-C was used, it was at relative low doses (5 or 10 J/m\(^2\)), which might give some apoptosis induction, but not very high. The alternative splices as described by Chandler et al. (2006) [47] were only seen at rather high doses of UV-C or cisplatin; these are in the same range as Moretti and colleagues used, but Chandler et al. did not investigate effects on protein levels. What can be noticed from that study is that while the dose of UV-C resulted in almost complete alternative splicing of \textit{mdm2} mRNA (full length is hardly detectable anymore), the main \textit{mdmx} mRNA expressed upon treatment with 50 J/m\(^2\) UV-C is still the full length mRNA. So, it is possible that under these ruthless conditions MDMX protein is still expressed.

What we do find somewhat surprising, though, is that apparently the induction of apoptosis by 40 J/m\(^2\) is largely dependent upon the targeting of p53 into the mitochondria. In several cellular systems it has been reported that UV-C (and cisplatin) can induce apoptosis in p53-null or p53-mutant cells [52, 63-65], indicating that wild-type p53 status is not a universal essential factor for UV-C or cisplatin-induced apoptosis. It will be interesting to see whether modulation of MDMX levels in p53-deficient cells also affects the UV-C or cisplatin-induced apoptosis.

In an attempt to understand and reconcile the various and sometimes contradicting results, a very well structured study should be performed in which one or two wild-type p53 expressing cell lines, if possible with their p53-deficient counterparts, were treated with various types of stress-inducing agents, mainly genotoxic agents like doxorubicin, cisplatin, IR and UV-C irradiation, but also with a Polymerase-II transcription inhibitor like \(\alpha\)-amanitin. Cells should be extensively analyzed at early and late time-points for expression of relevant genes (p53, MDM2, MDMX, p53-targets) at protein and mRNA level (including analyses of splicing variants) and for the biological outcome of the treatment. The latter can be done primarily by FACS analyses to determine DNA profile (PI staining), apoptosis (Annexin V staining), and DNA replication (BrdU incorporation). The
importance of MDMX regulation can be determined by using MDMX knockdown derivatives of the used cell lines. Such a study should be able to provide the answers to several questions regarding the regulation of MDMX by the various agents, and the importance of that for the biological response.

Role of MDMX in the ribosomal stress response: essential part of the DNA damage response?

The last years a strong link between nucleolar- and ribosomal stress and p53 activation has been established. Very importantly, Rubbi and Milner showed that disturbance of nucleolar integrity is important for the DNA damage response [66]. As such, they already showed that the DNA damage response and ribosomal stress are causally linked. Several groups subsequently showed that upon nucleolar disruption, ribosomal proteins are released, and several of those interact with MDM2 and prevent the MDM2-mediated degradation of p53 (recently reviewed by [67-68]). Very striking, none of the ribosomal proteins shown to interact with MDM2 interact with MDMX, suggesting a selective targeting by ribosomal proteins [69]. However, that does not imply that MDMX is not important for the ribosomal stress response. Indeed, these researchers show that upon treatment of cells with Actinomycin D or 5-FU, at concentrations that are reported not to induce a significant DNA damage response but only to give ribosomal stress, MDMX levels are downregulated. Importantly, that downregulation is needed for full p53 activation. The MDMX degradation is MDM2-dependent, but is not associated with increased phosphorylation of MDMXSerine367, so is distinct from the ATM/Chk2-stimulated MDMX degradation. Interestingly, the treatment with these compounds leads to release of L11 protein from the nucleoli, which binds MDM2 and inhibits MDM2-mediated degradation of p53, but stimulates the MDMX ubiquitination by MDM2 [69]. MDMX overexpressing cells showed an increased resistance to apoptosis induction by 5-FU, and indeed MDMX also inhibited the growth-suppressing effects of 5-FU on xenografts of HCT116 cells. Also in this case words of caution are needed. In the model tested, the HCT116 cells, the 5-FU effects are largely dependent to wild-type p53 function, as shown earlier [58]. However, other studies indicate that the effects of 5-FU on colorectal tumor cells are only very limited dependent on p53 status, and then only at really low concentrations [70]. Furthermore, in a small panel of 4 breast cancer cell lines, all expressing wild-type p53 and sensitive to Nutlin-3, only two showed a partial p53- dependence on growth inhibition by 5-FU, while one cell line actually is sensitized for 5-FU by reduced p53 levels [71]. The differential effects can possibly in part be explained by a difference in pRB status, which seems to dictate the dependence of 5-FU responsiveness on wild-type p53 status [72]. Therefore, it would be of
importance to investigate whether in other settings (e.g. p53 proficient, but pRB-deficient) MDMX levels also have an impact on 5-FU sensitivity.

A recent study from the Prives' lab showed another crosstalk between ribosomal proteins and MDM2/MDMX regulation of p53 [73]. They identified the small ribosomal subunit protein S7 as an MDM2-interacting protein, which can inhibit the MDM2-mediated degradation of p53. Interestingly, they found that S7 could only perform its function efficiently in the presence of MDMX. A putative involvement of S7 in MDM2-mediated degradation of MDMX was not investigated. A striking observation of these investigators was also that knockdown of either the ribosomal protein L11 or S7 attenuated the p53 activation not only by agents inducing ribosomal stress, but also DNA damaging agents like doxorubicin and neocarzinostatin. These data confirm the earlier mentioned observations by Rubbi and Milner, who already showed that DNA damage only affects p53 stability when it is causing nucleolar disruption [66].

**Alternative splicing of MDMX mRNA: what's its use?**

At the moment 6 different splicing variants of MDMX have been described, which are schematically depicted in figure 2. As mentioned before, the first reported splicing variant is the \textit{mdmx-S} mRNA, expression of which seemed to correlate with transformed cells and with normal cells stimulated to grow after serum-stimulation [48]. This was attributed to the fact that MDMX-S protein, which essentially comprises only the p53-binding domain (amino acids 1-114), and 26 unique C-terminal amino acids, is a stronger inhibitor of p53 as assayed by overexpression studies [49]. What is important to note is that the authors denote a protein of about 32 kDa in COS cells as the endogenous MDMX-S protein, which is significantly larger than the predicted molecular weight of approximately 16 kDa. The authors propose that this shift is caused by post-translational modification of the MDMX-S protein, but the type of modification was not determined. Strikingly, \textit{in vitro} translation of the \textit{mdmx-S} mRNA yielded also the 32 kDa protein, and a protein of about 17 kDa, which could represent the 'real' MDMX-S protein. We have recently obtained evidence that the 32 kDa protein is most likely not representing the true MDMX-S protein. Transfection of an MDMX-S expression vector, encompassing the total MDMX coding region but just lacking exon 6, with an N-terminal HA-tag, results in expression of a protein around 24 kDa detected with anti-HA antibody (Figure 4). Expression level of this protein is much lower than that of full length HA-MDMX protein upon transfection of same amount of plasmid DNA. In addition, smaller proteins around 18 kDa and 14 kDa are found, which are even lower expressed. However, we also made an expression vector in which we truncated the \textit{mdmx} cDNA at an XbaI restriction site, just downstream of the predicted stop codon of
MDMX-S protein in exon 7. So, this construct theoretically has the same coding potential. When this construct is expressed, only the smaller proteins of about 18 and 14kDa can be detected with anti-HA, again at low levels compared to full length HA-MDMX. Importantly, an antibody raised against the C-terminal unique amino acids of MDMX-S (Frank Bartel, personal communication) recognizes only the 18 kDa protein, identifying that as the real MDMX-S protein. The anti-MDMX antibody MX-82, with its epitope around amino acid 100 of MDMX, recognizes both the 18 kDa and 14 kDa protein, but not the 24 kDa protein. Two conclusions can be drawn from these results. First, the 24 kDa protein, possibly reflecting the 28 kDa protein mentioned by Rallapalli et al. [48], is most likely an artifact of the overexpression of the \textit{mdmx-S} cDNA, because it appears to lack the MX-82 epitope which should be included in the MDMX-S protein. Secondly, the smaller protein of about 14 kDa lacks a C-terminal part of MDMX-S; again most likely some artifact of the overexpression. Importantly, when we use same expression plasmids for \textit{in vitro} translation, we only detect the 18kDa protein, either with an anti-MDMX antibody (1328; raised against bacterially produced MDMX-S protein) or with anti-MDMX-S specific antibody (Figure 4). In addition, His6-tagged bacterially produced MDMX-S protein also has an apparent molecular weight of about 18 kDa.

As mentioned, we find that the MDMX-S protein is much lower expressed compared to full length MDMX, in contrast to previous reports, but confirming our previous finding [20]. This is not a result of transfection efficiency, as can be seen by the expression of the transfection control (Figure 4), and similar results were obtained after transduction of cells with lentiviral expression vectors for MDMX-S (not shown). Furthermore, real-time Q-PCR analysis showed that \textit{mdmx-S} mRNA was even higher expressed than full length \textit{mdmx} in these transfections (Figure 4B). Notably, treatment of transfected or transduced cells with proteasome inhibitor MG132 strongly increased the MDMX-S protein level, suggesting that the protein is very unstable. The levels of full length MDMX protein are only mildly increased upon MG132 treatment, while no effect is seen on the 24 kDa protein. Although we have no evidence at the moment, the rapid degradation of MDMX-S is possibly caused by the unfolded protein response (UPR); it is possible that this truncated MDMX protein is not properly folded, and as such is targeted via UPR for degradation.

In addition, we have put much effort in detecting the endogenous MDMX-S protein, which we expected not to be such a problem because the recently developed monoclonal antibody, MX-82, has its epitope within the MDMX-S region. However, even in cell lines that express more \textit{mdmx-S} mRNA compared to \textit{FL-mdmx} mRNA, only full length MDMX protein can be detected (data not shown). Therefore, we propose that the induction of
Functions of MDMX in the Modulation of the p53 Response

\(mdmx-S\) mRNA is a means of reducing full-length MDMX levels, and not so much the generation of a new MDMX protein with important physiological functions. This is also illustrated in figure 3, where it can be seen that full length \(mdmx\) mRNA decreases as \(mdmx-S\) mRNA levels increase upon various stress conditions, whereas MDMX-S protein levels are still undetectable (not shown).

**Figure 4:** Expression of Mdmx-S protein is much lower compared to full-length Mdmx and increased upon MG132 treatment, whereas mRNA levels are even higher in transfection studies. MCF7 cells were transfected with the indicated constructs (6-well plates; 1 \(\mu\)g of each construct/well; all wells were also transfected with 250 ng of CMV-eGFP expression vector as transfection control). Twenty four hrs after transfection, the cells were either mock treated or treated with 20 \(\mu\)M MG132 for 7 hours. Cell lysates were analyzed by Western Blotting with the indicated antibodies (6658 = MDMX-S specific antibody) and extracted RNA was analyzed by quantitative real-time RT-PCR.

The MDMX-A and MDMX-G variants were cloned from C33A cells; a somewhat exceptional cell line in that it expresses high levels of mutant p53 but also high levels of MDMX [30]. The corresponding MDMX proteins could not unequivocally be identified, no biological function has been determined, and whether this particular splicing event is
affected by any event, like stress conditions, is unknown. Therefore, these splicing variants will not be discussed here any further.

As mentioned above, UV-C irradiation was found to lead to alternative splicing of MDMX, resulting in X-Alt1 and X-Alt2. Again, no functional studies have been performed, synthesis of predicted alternative MDMX proteins has not been investigated, and the ratio between the full length MDMX and the alternative splicing variants is not known. Therefore, a biological relevance of these splicing variants is still hypothetical. Moretti and colleagues are the only group to report functional experiments on an endogenously expressed MDMX splice variant [74]. They identified an mdmx mRNA variant, which they called mdmx211, in a thyroid tumor cell line (ARO), which has structural similarity to the X-Alt2 variant (see Figure 2). The protein products produced by these variants both lack the major part of the p53-binding domain, but still contain the C-terminal domain including the RING finger. A difference is that the X-Alt2 variant contains all exon 11 encoded amino acids, including the Zn-finger, while the mdmx211 variant lacks the N-terminal part of the exon 11. Overexpression experiments indicate that MDMX211 protein can stabilize MDM2, and prevents the degradation of p53 by MDM2, reflecting what has been published about full length MDMX protein [75]. Overexpression of MDMX211 increased colony formation of both p53-wt and p53-negative cells, indicating a p53-independent oncogenic role of MDMX211.

Physiologically more relevant is the specific knockdown of this MDMX variant in ARO cells. Reducing levels of mdmx211 mRNA about 50% reduces the viability of this cell line, suggesting that both full length MDMX and the alternative MDMX211 form are contributing to the proliferation of these cells. However, the effects of specific knockdown of full length MDMX was not investigated in parallel, nor were relative levels of MDMX and MDMX211 proteins compared. Knockdown of MDMX211 did reduce MDM2 levels, but p53 levels or activity was not investigated, probably because these cells harbor a mutant p53. Again, these results are indicating a p53-independent oncogenic function of MDMX211. It is unfortunate that the authors have not studied the effect of specific knockdown of MDMX211 and full length MDMX in the FRO cell line, which appears to express similar levels of MDMX211, but contains a wild-type p53 gene [76]. The comparison between MDMX211 and FL-MDMX in this cell line would especially have been interesting in the experiment investigating the sensitivity to adriamycin-treatment.

As mentioned earlier, Moretti and colleagues published that overexpression of full length MDMX could increase the sensitivity to adriamycin [57], so one would predict that knockdown of MDMX would increase the number of viable cells after adriamycin-treatment. This would then indeed be opposite from the effects of knockdown of
MDMX211 in ARO cells, which increases the sensitivity to adriamycin in these thyroid tumor cells [74]. All in all, these results indicate that MDMX-211 has oncogenic potential, but whether that is increased compared to full length MDMX is not known at the moment.

A number of studies investigated the expression levels of *mdmx* mRNA and of *mdmx* splicing variants in a panel of tumors. An analysis of malignant gliomas showed an amplified *mdmx* gene in a subset of tumors, none of which had p53 mutation. Interestingly, ratio between *mdmx*-S/*mdmx*-FL mRNA was found to be significantly higher in grade IV glioblastomas compared to grade III anaplastic gliomas or grade II gliomas [77]. Bartel and colleagues studied 66 soft tissue sarcomas for *mdmx* gene amplification and *mdmx*-FL/*mdmx*-S mRNA expression [78]. They also found an association between tumor grade and overexpression of the *mdmx*-S splice form. Furthermore, from their analyses they concluded that the *mdmx*-S is a prognostic factor that predicts a poor outcome and a significantly shortened survival time for STS patients. No correlation between p53 status and *mdmx*-S expression was evident.

Keeping in mind that our analyses so far suggest that increase in the ratio of *mdmx*-S mRNA compared to *mdmx*-FL mRNA is more a means to decrease full length *mdmx*, it could imply a tumor suppressor function for the MDMX protein, as is also suggested from the study of Moretti and colleagues [79]. They found that in 57 papillary thyroid tumors, which all contain a wild-type p53 gene, a decrease of full length MDMX is associated with tumor stage (stage 1 versus others), indicating downregulation of MDMX protein levels during tumor progression.

An increased ratio *mdmx*-S:*mdmx*-FL (ratio ≥1.5) mRNA is found in a significant percentage of these thyroid tumors, but is not associated with any clinical data. Expression of the *mdmx*211 mRNA is also found in a subset of tumors, again not correlating with any patient data. Together, these data on the expression of *mdmx*-FL and *mdmx* mRNA splicing variants in combination with other analyses of *mdmx* gene amplification, *mdmx* mRNA expression and MDMX protein analyses in tumors [80-83] strongly indicate that the function of MDMX in tumorigenesis is strongly dependent on the tumor type. Although in several tumor types MDMX appears to have an oncogene function, in others it seems to be anti-oncogenic. So far the study by Moretti and colleagues has been the only indicating a tumor suppressor role of MDMX in wild-type p53 expressing human tumors; unfortunately, the authors do not show any data on the levels of p53 in these tumors. Possibly, the p53 gene is still wild-type but the expression of the p53 mRNA is silenced yielding an essential p53-null tumor. This is important in the light of other publications in mouse models indicating an anti-proliferative function of MDMX [84-85]. In the first study a growth suppressive function of mouse MDMX in a p21<sup>WAF1</sup>-deficient background was observed
[84]. In addition, in a p53-null background loss of MDMX increased the growth of mouse embryo fibroblasts and endogenous levels of MDMX suppressed tumorigenicity in p53-null mice [85]. In p53-null cells MDMX prevents the chromosome loss in hyperploid cells by inhibiting spindle multipolarity, in that way stimulating genome stability. Thus, under certain conditions MDMX indeed can function as an antioncogene. It would be interesting to investigate whether the reduced MDMX expression in later stage thyroid tumors [79] is correlated with low expression of p21WAF1 and/or aneuploidy.

Recently two mouse models have been published that overexpress an *mdmx* transgene. Unfortunately, the mice show different phenotypes, which cannot easily be explained at the moment. In the first model, the generally overexpressed MDMX protein induces spontaneous tumor formation, mostly sarcomas, indicating an oncogene function of MDMX [86]. MDMX overexpression also strongly accelerated the tumor development of p53 heterozygous mice. Unfortunately, the effect of MDMX overexpression was not investigated in a p53-null background. In the light of the study of Jones and colleagues [85], showing that loss of MDMX in p53-null mice accelerates tumor formation, one might predict that overexpression of MDMX should reduce the rate of spontaneous tumor formation in the p53-null mice.

Surprisingly, the second *mdmx* transgenic mouse model, in which a Myc-tagged MDMX is overexpressed, does not accelerate tumor formation, neither the spontaneous nor the Eμ-Myc induced tumorigenesis [87]. Interestingly, only a heterozygous myc-mdmx transgenic mouse is viable; the homozygous transgenic mice die during embryogenesis in a p53-independent manner, due to massive vascular maturation defects. An in-depth side-by-side comparison between the two *mdmx* transgenic mouse models could possibly explain the observed differences in phenotypes.

In conclusion, most available data suggest that the human *mdmx* gene functions as an oncogene when overexpressed in tumors still expressing wild-type p53, which is supported by most mouse models. However, the question whether expression of MDMX or alternative MDMX proteins produced by alternative splicing has an oncoprotein or a tumor suppressor protein function in tumors lacking p53 (or p21) or expressing a mutant p53 has still to be answered.

**Transcriptional regulation of MDMX expression**

Up till recently it was only Jiandong Chen and colleagues who investigated regulation of transcription initiation of the *mdmx* gene [88]. They found that, like the *mdm2* P2 promoter, mitogenic signaling stimulates transcription of the *mdmx* promoter. Especially two Ets-family binding sites are important in that respect, and the MEK/ERK signaling pathway was found to be involved in the induction of MDMX promoter activity upon growth factor
stimulation. Expression of oncogenic K-Ras also activated the MDMX promoter. Importantly, in a panel of colon tumors they find an increasing MDMX expression with tumor stage, and this increased expression of MDMX does correlate to some extent with increased activity of the MAPK pathway as measured by phospho-ERK staining. It is intriguing to note that around 50% of later stage colon tumors stain positive for MDMX in this study, and that approximately 50% of colon tumors contain p53 mutations. It would have been interesting had the authors determined the p53 status of the samples they used for MDMX staining to investigate a putative -negative- correlation.

These results indicate that enhanced mitogenic signaling in tumor cells which have lost p14ARF expression might inhibit the tumor suppressor activity of p53 by enhancing both **mdmx** and **mdm2** gene transcription [88-89].

More recently, a new twist to the p53/MDM2/MDMX interactions has been added. Wei and colleagues had reported that the intron 1 of the human **mdmx** gene contains a p53RE, as identified in a global p53 ChIP experiment [90]. Li et al. were the first to publish that this p53RE indeed mediates transcriptional activation of the **mdmx** gene upon activation of p53 by Nutlin-3 [91]. Furthermore, they provide evidence that reducing MDMX levels in the TGCT (testicular germ cell tumor) cell line NT2/D1 induces low levels of apoptosis, but strongly sensitizes these cells for growth inhibition by Nutlin-3. However, this effect was not observed in other TGCT cell lines tested. The authors do suggest that in the NT2/D1 cells MDMX is involved in a negative feedback loop, similar to MDM2. These studies do not distinguish between the importance of the constitutive **mdmx** transcription and the p53-induced transcription. In collaboration with the groups of Jeremy Blaydes and Frank Bartel we also recently reported that the previous identified p53RE in the first intron of **mdmx** is directing p53-induced transcription of the **mdmx** gene [92]. In addition to the earlier publication we showed that the p53RE initiates transcription from an alternative promoter (**mdmx** P2), which includes an alternative first exon (named exon 1β) in the **mdmx** mRNA. This exon contains an in-frame upstream ATG relative to the canonical first ATG, which results in the synthesis of 18 extra N-terminal amino acids. It was found that the **mdmx** P2 promoter can be induced in almost all cells tested that express wild-type p53 (only a few primary human cell cultures showed a very low or undetectable expression). Furthermore, every type of p53 activation (Nutlin-3, DNA damage, ribosomal stress, Leptomycin B, activation of p14ARF expression) increased transcription from the **mdmx** P2 promoter. Most importantly, the inclusion in the **mdmx** P2 mRNA of a unique exon provided us with the opportunity to investigate specifically the contribution of the p53-induced **mdmx** mRNA expression in the p53 response. Indeed, shRNA targeting sequences in the unique exon 1β increased the p53 response upon treatment of cells with Nutlin-3 or DNA damage.
Chapter 2

(etoposide) and enhanced the p53-induced growth inhibition. These results together strongly establish *mdmx* as a p53 target gene, involved in a negative feedback loop to attenuate the p53 response.

Conclusions and future perspectives

When the identification of the *mdmx* gene was presented in 1996 [18], the p53 field was not immediately impressed. The added value of MDMX to MDM2 could not be recognized. MDM2 was expected to be sufficient to keep p53 in check, and to regulate the p53 response. However, the p53-dependent embryonal lethality of *mdmx* knockout mice established MDMX as an essential regulator of p53 activity. In more recent years it has become clear that during development MDM2 is the more general regulator of p53 activity. However, the intricate regulation of MDMX has been shown to be essential for a controlled p53 response upon various forms of stress, as much as MDM2 regulation. Although great insight into regulation of MDMX expression and activity has been obtained, much has still to be learned. How is MDMX degraded upon ribosomal stress, since DNA-damaged induced phosphorylations are not involved? How is the alternative splicing of MDMX upon certain types of genotoxic stress regulated? Do the various identified *mdmx* mRNAs encode proteins with important biological functions in the coordination of stress response or in tumorigenesis? Under which circumstances is MDMX acting as an oncogene, and when is it stimulating the p53 tumor suppressor response? The answers on these questions will be important for understanding the role of MDMX in tumorigenesis, and for obtaining more insight into the possibility to regard MDMX as a target for therapeutic intervention in cancer.

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Functions of MDMX in the Modulation of the p53 Response

References

Chapter 2


Functions of MDMX in the Modulation of the p53 Response


Chapter 2


Functions of MDMX in the Modulation of the p53 Response


c-Abl phosphorylates Hdmx and regulates its interaction with p53

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Chapter 3

Abstract
Upon exposure to DNA damage the p53 tumor suppressor is accumulated and activated to stall cellular growth. For this to occur, p53 must be relieved from its major inhibitors, Mdm2 (Hdm2 in humans) and Mdmx (Mdm4; Hdmx in humans). A key mechanism controlling this relief is the post-translational modifications of p53 and its inhibitors. We have previously demonstrated that the stress-activated tyrosine kinase, c-Abl, contributes to the relief of p53 from Hdm2. Because Hdmx is the major inhibitor of p53 activity, the additional possibility that c-Abl protects p53 through targeting Hdmx was explored in this study. c-Abl was found to interact with and to phosphorylate Hdmx. This phosphorylation was enhanced in response to DNA damage. Importantly, we mapped the sites of phosphorylation to the p53 binding domain of Hdmx. One of these phosphorylations, on tyrosine 99, inhibited Hdmx interaction with p53. This inhibition is consistent with the predicted role of this residue in the interaction with p53 based on the crystal structure of the interaction site. Our results show that c-Abl not only targets Hdm2, but also Hdmx, which together contribute to p53 activation in response to DNA damage.

The abbreviations used are:
wt, wild type;
HA, hemagglutinin;
KD, kinasedefective;
aa, amino acids;
IP, immunoprecipitation;
ATM, ataxia telangiectasia mutated;
GFP, green fluorescent protein;
BD, binding domain.
Introduction

Tight regulation of the p53 protein is critical for tumor suppression, for a proper cellular response to stress, and for mouse development (1). P53 stability and activity are regulated by two major inhibitors, Mdm2 and Mdmx (Mdm4; Hdmx in humans), respectively. The importance of the Mdm genes as p53 regulators was best exemplified by the demonstration that elimination of p53 fully rescues \textit{mdm2} and \textit{mdmx} null embryos from lethality (2). Mdm2 acts as the direct E3 ligase of p53, promoting it for proteasomal degradation and nuclear export (3). Unlike Mdm2, Mdmx lacks detectable ubiquitin E3 ligase activity and, hence, does not target p53 for degradation but, rather, inhibits p53 transcriptional activity by binding to its transactivation domain (for review, see Ref. 4). Down-regulation or mutations in Mdmx increase the induction of p53 target genes without a change in p53 expression levels (4, 5). The importance of Mdmx as a regulator of p53 is further demonstrated by its link to cancer development. Hdmx is overexpressed or amplified in 10–20% of human cancers including breast, stomach, colon, and lung cancers (for review, see Ref. 6) and in 65% of retinoblastomas (7). Interestingly, in papillary thyroid carcinomas Hdmx expression is down-regulated, although certain Hdmx variants with p53 inhibitory properties were abnormally expressed in these tumors (8). Importantly, overexpression of Mdmx correlates well with a normal status of p53, supporting the notion that this inhibitory pathway is sufficient to suppress p53 and, thus, negates the need for p53 mutation. Thus, abrogating Mdmx function may be an important approach for reactivating p53 in cancer cells (for review, see Ref. 2).

Mdm2 and Mdmx form homo- and heterodimers through their RING domains (9, 10); the ratio of these forms affect their protein stability and the extent of p53 inhibition (4, 11). An important link between the two proteins has been reported under stress conditions. After DNA damage, Mdmx accumulates in the nucleus (11, 12) where it inhibits p53 activity (4, 13), and it is subsequently degraded in an Mdm2-dependent manner (14–16). Mdmx degradation together with the self-degradation of Mdm2 contributes considerably to p53 activation and stabilization upon stress (for review, see Ref. 17). An important mechanism leading to p53 activation in response to DNA damage involves post-translational modifications, which are mediated by several upstream positive regulators (for review, see Ref. 18). One such activator is the c-Abl non-receptor tyrosine kinase (19). c-Abl has been implicated in the cellular response to stress by promoting cell cycle arrest or apoptosis (20, 21). c-Abl is critical for the accumulation of p53 in response to DNA damage (22). c-Abl activates p53 by neutralizing the inhibitory effects of Hdm2 (22–25). Given the important inhibitory role of Mdmx in p53 regulation and the link between c-Abl and Hdm2, we asked whether c-Abl also protects p53 through targeting Hdmx. We report here a physical and biochemical link between c-Abl and Hdmx. c-Abl interacts with and phosphorylates Hdmx.
Chapter 3

These phosphorylations are enhanced in response to stress. Tyrosine phosphorylations of Hdmx were mapped to the p53 binding domain of Hdmx, and one of these phosphorylations inhibits p53 binding. Overall, our results reveal that Hdmx is a new target of c-Abl, which together with Hdm2 contributes to p53 activation in response to stress.

Experimental Procedures

Cell Lines, Transfection Assays, and Cell Treatments—H1299 lung adenocarcinoma cells, lacking p53 expression, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. 293 and 293T human embryo kidney epithelial cells, MCF-7 breast carcinoma cells, and mdm2/p53 double null (2KO) fibroblasts were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected by the calcium phosphate method as described previously (26). Transfections using polyethyleneimine (PEI; Sigma) were done by mixing DNA with PEI reagent in serum-free medium for 5 min. 1 µl of polyethyleneimine (1 mg/ml) was used for 1 µg of DNA. The polyethyleneimine-DNA complex was applied to the cells for 5–7 h before the medium was replaced. Transfections with FuGENE 6 (Roche Applied Science) were performed in 9-cm dishes according to the manufacturer’s instructions. The FuGENE 6-DNA complex was applied to the cells for 24 h before the medium was replaced. A constant amount of plasmid DNA in each sample was maintained by supplementing with empty plasmid. Usually, cells were transfected with 5–8 µg of the indicated plasmid for a 9-cm plate and with 2–5 µg for a 5-cm plate. For measuring the cellular response to stress, cells were treated with 3mM H2O2 (Merck, Germany) together with 1mM sodium orthovanadate (Sigma) for the indicated times. To induce DNA damage, cells were treated with either 2–2.5 g/ml doxorubicin hydrochloride (Sigma) or 500 ng/ml neocarzinostatin (Sigma) in the dark. To inhibit proteasomal degradation, the proteasome inhibitor MG132 (Sigma) was added for 4 h.

Plasmids, in Vitro Site-directed Mutagenesis, and Generation of Lentivirus—Expression plasmids used in this study were human wild-type (wt) p53 (pRC-CMV-p53), mouse wt non-tagged and HA-tagged c-abl (pCMV-c-abl, type IV), mouse kinase-defective (KD) c-abl (pCMV-c-ablK290H, type IV) (24), p53-responsive cyclin G-luciferase (26), and an empty vector (pCMV-Neo-BamHI). Hdmx vectors were pcDNA3.1 human HAHdmx, pcDNA3.1 human wt hdmx, HA-hdmx (amino acids (aa) 1–318 followed by 25 alternative aa), hdmx-D (aa 1–206 followed by 13 alternative aa), HA-hdmx-G (Δ27–124 aa). Hdmx deletion mutants were Myc-tagged hdmx ΔN (aa 101–490) and hdmx p53 binding domain (p53 BD)-His-Myc (aa 1–153). The Tyr to Phe substitutions within Hdmx were generated by using the QuikChange site-directed in vitro mutagenesis kit (Stratagene) on the full-length and the p53 BD hdmx forms. For the production of GFP-c-Abl lentivirus, the following vectors were used; GFP-c-Abl was cloned into SIN18.hEF1-WPRE vector using SmaI and XbaI sites, pCMV_R8.91-packaging construct, and pMD2.VSVG envelope construct. For the production of lentivirus these three vectors were co-transfected into HEK293T cells, and lentivirus was collected from the supernatants after 48 h. For the production of si-3_UTR-Hdmx lentivirus (MISSION Library Sigma-Aldrich) the manufacturer’s instructions were followed.

Antibodies—Anti-Hdmx antibodies used were: rabbit polyclonal sera p55, p56 (27), and 1328 (P. de Graaf and A. G. Jochemsen, unpublished data), mouse monoclonal antibodies 6B1A, 11F4D, and 12G11G (28), and mouse monoclonal MX-82 antibody (Sigma MX-82). Anti-c-Abl antibodies used were the rabbit polyclonal K-12 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the monoclonal Abl-148 antibody (Sigma); the anti-phospho-Hdmx antibodies were phospho-Tyr-55 (Sigma PH-MDMX-55) and phospho-Tyr-99 monoclonal antibodies (Sigma PH-MDMX-169) and anti-phospho-Ser-367 rabbit polyclonal antibody (29). Additional antibodies used were: anti-human p53 monoclonal antibodies PAb1801 and DO1; anti-p53 goat polyclonal
c-Abl phosphorylates Hdmx and regulates its interaction with p53

antibody (FL-393 Santa Cruz Biotechnology); anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz Biotechnology); anti-phospho-p53 Ser-20 antibody (Cell Signaling Technology, Inc., Danvers, MA); anti-Hdm2 SMP14 and 2A9 monoclonal antibodies, anti-GFP (Roche Applied Science), anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-14-3-3 tau (BIOSOURCE, Camarillo, CA), horseradish peroxidase-conjugated affinity-purified goat anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA), and EnVision peroxidase anti-mouse or anti-rabbit IgG antibodies (DAKO Corp., Glostrup, Denmark).

Immunoblotting, Immunoprecipitation, in Vivo Kinase Assay and Nickel Pulldown Assay—Western blot analysis and immunoprecipitation assay were performed as previously described (26), whereas the lysis buffer for immunoprecipitation consisted of 50 mM Tris, pH 8.0, 150–300 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 4 mM NaF, and 1 mM sodium orthovanadate. Nickel pulldown and in vivo kinase assay were performed as described previously (24).

Luciferase Assay—This assay was done essentially as previously described (24).

Detection of Phosphorylated Tyr by Mass Spectrometry—Phosphorylated and non-phosphorylated Hdmx proteins were derived from in vivo kinase assay using wt or kinase-defective c-Abl. The proteins were digested using Asp N enzyme, and mass spectrometry was carried out with Qtof2 (Micromass, England) using nanospray attachment (30). Data analysis was done using the biolynx package (Micromass, England), and data base searches were performed with the Mascot package (Matrix Science).

RESULTS

Interaction between c-Abl and Hdmx
To determine whether the protection of p53 by c-Abl involves relief from Hdmx, we examined a possible interaction between c-Abl and Hdmx proteins in cultured cells. HEK293 cells were transfected with expression plasmids for HA-Hdmx either alone or together with wt or a KD c-AblK290H mutant of c-Abl. Forty-eight hours post-transfection, cell extracts were subjected to immunoprecipitation (IP) using anti-c-Abl Abl-148 antibody followed by immunoblotting (IB) with anti-Hdmx antibodies. As shown in Fig. 1A, Hdmx was efficiently co-precipitated only in the presence of c-Abl, demonstrating an interaction between the two proteins in cultured cells. The interaction between Hdmx and the KD mutant of c-Abl appeared to be weaker. To further establish the interaction between Hdmx and c-Abl, we performed a reversal experiment where IP was done with anti-Hdmx 6B1A antibody and IB with anti-c-Abl antibody. Hdmx efficiently co-precipitated c-Abl (Fig. 1B). These results demonstrate an interaction between the two proteins in cultured cells. Next, we investigated whether endogenous Hdmx interacts with endogenous c-Abl. To answer this question, we performed co-immunoprecipitation experiments using MCF-7 cell line expressing relatively high endogenous Hdmx levels. c-Abl was immunoprecipitated from cell extracts using anti-c-Abl K-12 antibody followed by immunoblotting with anti-Hdmx antibody. As shown in Fig. 1C, Hdmx protein was co-immunoprecipitated by c-Abl, providing evidence for an interaction between the two endogenous proteins.
FIGURE 1. Hdmx interacts with c-Abl in vivo. A, B, E, and F, HEK293 cells were transfected with expression plasmids for \textit{c-abl} or \textit{c-abl}KD(K290H; panel A only) either alone or together with the indicated \textit{hdmx} expressing plasmids. Forty-eight hours post-transfection cell extracts were subjected to IP analysis using the following antibodies: anti-c-Abl monoclonal ABL-148 antibody (A), anti-Hdmx 6B1A antibody (B), anti-c-Abl K-12 polyclonal antibody (E and F). IP was followed by IB with anti-Hdmx antibody (MX-82) (A and F) or 6B1A (E) or with anti-c-Abl antibody (ABL-148) for B. c-Abl levels were monitored by blotting with anti-c-Abl antibody (ABL-148). C, MCF-7 cells were subjected to IP analysis as described for F. Note that shorter exposure for Hdmx is presented in input panel as compared with co-immunoprecipitation panel. D, schematic representation of the Hdmx deletion mutants used in this study. All the mutants were found to bind c-Abl. Amino acid composition of the mutants is indicated on the right; note that mutant C and D contains an additional 25 or 13 residues, respectively.
c-Abl phosphorylates Hdmx and regulates its interaction with p53

Both c-Abl and Hdmx interact with p53 and Hdm2 (19). We, therefore, investigated whether the c-Abl/Hdmx interaction is mediated by p53 and/or Hdm2. To address this question, HEK293 cells were transfected with expression plasmids for c-Abl in combination with different Hdmx deletion mutants. First, we tested the involvement of p53 by using Hdmx-G, a product of an alternative splice variant that lacks amino acids 27–124 encompassing the p53 binding domain of Hdmx (Ref. 15 and Fig. 1D). Twenty-four hours posttransfection cell extracts were subjected to immunoprecipitation assay. As shown in Fig. 1E, Hdmx-G (ΔN) bound efficiently to c-Abl, suggesting that p53 is not essential for c-Abl-Hdmx interaction under these conditions. Next we investigated the contribution of Hdm2 to the interaction by using another Hdmx alternative splice variant, Hdmx-D, which lacks the C terminus (amino acids 207–490) including the Hdm2 binding region and contains an additional 13 alternative amino acids after amino acid 206 (Fig. 1D). As shown in Fig. 1F, Hdmx D (ΔC) efficiently co-precipitated with c-Abl, implying that the Hdm2 binding domain is not critical for c-Abl-Hdmx interaction. To further define the interaction region of c-Abl within Hdmx, a series of additional deletion mutants of Hdmx (Fig. 1D) were tested using the same assay. All the Hdmx deletion mutants tested were able to bind c-Abl (supplemental Fig. S1), implying that c-Abl may bind Hdmx at more than one site and/or that additional proteins are involved in the interaction with some of these mutants.

c-Abl Phosphorylates Hdmx within the p53 Binding Domain

Because c-Abl is a tyrosine kinase, we asked whether Hdmx is phosphorylated by c-Abl. There are nine Tyr residues within Hdmx, five of which are clustered within the p53 BD (Fig. 2A) and are conserved among mice, rat, and human (Fig. 2B). In view of the possible link to p53 regulation, we examined the ability of c-Abl to phosphorylate an Hdmx deletion mutant containing the N-terminal p53 BD (amino acids 1–153) in an in vivo kinase assay. HEK293 cells were transfected with expression plasmids for the Myc- and His-tagged hdmx p53 BD either alone or in combination with wt or c-Abl KD. Twenty-four hours post-transfection Hdmx was isolated from cell extracts using nickel resin, and Tyr phosphorylation of Hdmx was monitored with anti-phosphotyrosine antibody. As shown in Fig. 2C, Tyr phosphorylation of Hdmx p53 BD was detected only in the presence of wt c-Abl, supporting a role for c-Abl in Tyr phosphorylation of Hdmx within the p53 BD.
FIGURE 2. c-Abl-dependent tyrosine phosphorylation of Hdmx.

A, schematic representation of the tyrosine residues in Hdmx. The cluster of the tyrosine residues within p53 binding domain of Hdmx is highlighted. B, alignment of the Mdmx amino acid sequence from rat, mouse, and human. The conserved tyrosine residues within Mdmx are highlighted. C, HEK293 cells were transfected with expression plasmids for c-abl or c-abl KD either alone or together with His- and Myc-tagged hdmx p53 BD (binding domain; amino acids 1-153). Twenty-four hours post-transfection cells were harvested, and Hdmx p53 BD was isolated from cell extracts using nickel resin. Phosphorylation was detected with anti-phosphotyrosine antibody (4G10), and Hdmx levels were monitored by blotting (IB) with anti-Myc antibody. The expression level of c-Abl was monitored with anti-c-Abl antibody (ABL-148).

Tyr-99 and Tyr-55 Are the Major Sites within the p53 BD of Hdmx

To identify the c-Abl Tyr phosphorylation site(s) within the p53 BD of Hdmx, we employed mass spectrometric analysis along with site-directed mutagenesis. Hdmx p53 BD was phosphorylated in vivo by c-Abl (Fig. 2C) and then subjected to mass spectrometry analysis. Tyr-55 was identified as a potential Tyr phosphorylation site (data not shown). Additionally, we generated substitution mutants from Tyr(Y) to Phe(F) for each Tyr residue in the p53 BD of Hdmx, either alone or in various combinations. The effect of these substitutions on Hdmx phosphorylation by c-Abl was measured in phosphorylation assays in vivo. HEK293 cells were transfected with expression plasmids for wt hdmx p53 BD or phosphorylation mutants together with wt c-Abl or c-Abl KD. Twenty-four hours posttransfection, Hdmx was precipitated, and Tyr phosphorylation analysis revealed that substitutions of Y99F and Y55F, but not the other Tyr residues within the p53 binding domain, reduced the extent of Hdmx phosphorylation (Fig. 3A and data not shown). Tyr phosphorylation was almost abolished in the Hdmx Y55F,Y99F double mutant (Fig. 3A), supporting the notion that Tyr-55 and Tyr-99 are the major c-Abl phosphorylation sites.
c-Abl phosphorylates Hdmx and regulates its interaction with p53

within the p53 BD of Hdmx. Among these two sites, Y99F substitution had a greater effect on Hdmx phosphorylation (Fig. 3A); hence, it was assumed to be a more dominating site. To further verify the phosphorylation of Hdmx on Tyr-99 and Tyr-55, anti-phospho-monoclonal antibodies were raised. To determine the specificity of these antibodies, HEK293 cells were transfected with expression plasmids for the Myc and His-tagged hdmx p53 BD in combination with wt c-Abl or c-Abl KD. Specific c-Abl-induced Hdmx phosphorylations on Tyr-55 and Tyr-99 were detected with the corresponding anti-phospho antibodies (Fig. 3B). A similar result was obtained when the experiment was repeated with full-length non-tagged wt Hdmx (Fig. 3C).

**FIGURE 3.** c-Abl phosphorylates Hdmx on Tyr-99 and Tyr-55 in vivo. A, HEK293 cells were transfected with the indicated plasmids. Twenty-four hours later Hdmx p53 BD was isolated by nickel resin from cell extracts, and the protein complex was subjected to Western blotting (IB) using the indicated antibodies as described in Fig. 2C. B and C, HEK293 cells were transfected with the indicated plasmids. Twenty-four (B) or forty-eight (C) hours later cell extracts were analyzed by Western blotting using anti-phospho-Tyr-55 antibody and anti-phospho-Tyr-99 antibody. Total Hdmx levels were monitored by blotting with anti-Hdmx 1328 polyclonal antibody. The position of Tyr-55 and Tyr-99-phosphorylated Hdmx and total Hdmx is indicated. A dividing line in B indicates that lanes were merged from different parts of the same gel.

Phospho-Tyr-99 Affects the Phosphorylation of Tyr-55

Because the phosphorylation sites reside within the same domain of Hdmx, it was of interest to test a potential dependence between the two sites. To examine this possibility, HEK293 cells were transfected with expression plasmids for Myc and His-tagged wt hdmx p53 BD or phosphorylation mutants either alone or in combination with wt c-Abl or c-Abl KD. The phosphorylation of Tyr-99 was detected in wt Hdmx and the Y55F substitution mutant (Fig. 4A), suggesting that the Tyr-99 phosphorylation is independent of Tyr-55 phosphorylation. Interestingly, the phosphorylation of Tyr-55 was reduced not only in the Y55F mutant but partially also in the Y99F mutant (Fig. 4B). A similar effect was observed with full-length non-tagged Hdmx (Fig. 4C). This suggests that phosphorylation on Tyr-99 is required at least partially for an efficient phosphorylation of Tyr-55. An alternative explanation is that the Y99F substitution impairs the interaction between Hdmx and c-Abl. To test this possibility HEK293 cells were transfected with expression plasmids for c-Abl.
either alone or together with Myc-His-tagged wt or Y99F hdmx p53 BD mutant. Hdmx was immunoprecipitated using anti-Myc antibody, and the amount of co-precipitated c-Abl was determined by IB using anti-c-Abl antibody (Fig. 4D). As shown in Fig. 4D, the Y99F substitution did not reduce the c-Abl-Hdmx interaction but, rather, increased it.

FIGURE 4. Tyr-99 is required for the efficient phosphorylation of Tyr-55, and both tyrosine phosphorylations are independent of Ser-342 and Ser-367 phosphorylation. A-C, HEK293 cells were transfected with the indicated plasmids. Twenty-four (A and B) or forty-eight hours (C) later cell extracts were subjected to Western blotting using either anti-phospho-Tyr-55 antibody (B and C) or anti-phospho-Tyr-99 antibody (A and C). Total Hdmx levels were monitored using anti-Hdmx 1328 polyclonal antibody. The position of Tyr-55 and Tyr-99-phosphorylated Hdmx and total Hdmx is indicated. Hdmx p53 BD mutant was described in Fig. 2C. D, HEK293 cells were transfected with the expression plasmid for c-abl alone or together with Myc and His-tagged hdmx p53 BD either wt or Y99F mutant. Twenty-four hours post-transfection Hdmx was immunoprecipitated using anti-Myc antibody. The level of the co-precipitated c-Abl was monitored by blotting with anti-c-Abl antibody (ABL-148). The expression level of the Hdmx was investigated by using anti-Hdmx antibody. E, HEK293-T cells were transfected with expression vectors for HA-tagged hdmx or hdmx-S342/367A or empty vector together with c-abl. Cells were either non-treated or treated with 3 mm H2O2 for 45 min together with tyrosine phosphatase inhibitor sodium orthovanadate (Na3VO4; 1 mm) for 5 h before harvest. Twenty-nine hours post-transfection cell extracts were subjected to IP with anti-HA rabbit polyclonal antibody. Immunoprecipitates were analyzed by Western blotting with anti-phospho-Tyr-55-Hdmx, anti-phospho-Y99-Hdmx, and anti-Hdmx MX-82 to monitor total Hdmx levels. To show the absence of Ser-367 phosphorylation of the mutant, the same protein extracts were used in immunoprecipitation with anti-HA monoclonal antibody, and immunoprecipitates were analyzed on Western blot with anti-phospho-Ser-367-Hdmx or anti-Hdmx antibody (BL1258).
Interplay between c-Abl-mediated Phosphorylation of Hdmx and the DNA Damage-induced Hdmx Phosphorylation on Ser-342, Ser-367, and Ser-403 and 14-3-3 Binding

It has been shown that Hdmx is independently phosphorylated on Ser-342, Ser-367, and Ser-403 in response to double strand breaks by ATM (ataxia telangiectasia mutated) or by its target Chk2 (29, 31). Each of these phosphorylations contributes to the increased ubiquitination and degradation of Hdmx after DNA damage, caused by dissociation of the herpes virus-associated ubiquitin-specific protease (HAUSP) from both Hdmx and Hdm2 and promotion of nuclear accumulation of Hdmx (12, 32). Phosphorylation of Ser-342 and Ser-367 was shown to be essential for creating a binding site for several isoforms of the 14-3-3 protein, leading to nuclear localization of Hdmx, and promoting the degradation of Hdmx (29, 33, 34). Because c-Abl is also a target for ATM-dependent phosphorylation after DNA damage, a possible interdependence between the serine and tyrosine phosphorylations on Hdmx was studied. Initially, we asked whether phosphorylation of Hdmx Tyr-55 and Tyr-99 depends on the phosphorylation of Hdmx-Ser-342 and Ser-367. The extent of c-Abl-mediated phosphorylation of Hdmx-Tyr-55 and Hdmx-Tyr-99 was compared between Hdmx S342A/S367A mutant and wild-type Hdmx. We found that the S342A/S367A mutant was still phosphorylated on both Tyr-55 and Tyr-99, indicating that the Tyr-55 and Tyr-99 phosphorylations are independent of Ser-342/Ser-367 phosphorylation (Fig. 4E).

Next, we investigated if phosphorylation of Ser-367 depends on phosphorylation of Hdmx-Tyr-99. To this end, Ser-367 phosphorylation was compared between wild-type Hdmx and Hdmx-Y99F mutant. As can be seen, no significant difference was observed in the Ser-367 phosphorylation between wildtype and Y99F mutant of Hdmx (supplemental Fig. S2A). Another way of approaching this question is to measure whether the Y99F and Y55F substitutions would affect the increased interaction between Hdmx and 14-3-3 proteins upon induction of double strand break by neocarzinostatin. This interaction is dependent on Ser-367 and Ser-342 phosphorylation (29). We found that both Hdmx-Y55F and Y99F mutants were able to bind 14-3-3 tau and show increased binding similar to wt Hdmx after DNA damage, whereas Hdmx-S367A was unable to interact with 14-3-3 tau as previously shown (supplemental Fig. S2B). These results are further supported by the preliminary observation that the Y55F and Y99F mutants indeed accumulate in the nucleus, as does wild-type Hdmx, upon treatment of cells with neocarzinostatin (data not shown). Together these results show that phosphorylation of Hdmx-Ser-367 is independent of Hdmx-Tyr-55 and -Tyr-99 phosphorylation.
Phosphorylation of Hdmx on Tyr-99 but Not on Tyr-55 Impairs Its Interaction with p53

The location of Tyr-99 and Tyr-55 within the p53 binding domain of Hdmx begged the question as to whether the phosphorylation of these tyrosines modulates the p53-Hdmx interaction. To address this question, H1299 cells, lacking p53 expression, were transfected with expression plasmids for p53, either alone or together with hdmx p53 BD and c-Abl. Twenty-four hours after transfection, cell extracts were subjected to IP using anti-p53 antibody followed by immunoblotting with either specific anti-phospho- antibodies or anti-Hdmx antibodies. As expected, p53 interacts efficiently with Hdmx. By marked contrast, p53 interaction with the Tyr-99 phosphorylated form of Hdmx was not detected, even after very long exposure of the film (Fig. 5A). This result suggests that Tyr-99 phosphorylation inhibits the interaction of Hdmx with p53. In contrast to Tyr-99, the phosphorylation of Tyr-55 did not impair its interaction with p53 (Fig. 5B) and in some experiments even enhanced it (supplemental Fig. S3). To further establish these findings, we monitored the effect of Hdmx tyrosine phosphorylations at more physiological levels of expression. We have generated MCF-7 stable lines expressing the following full-length HA-tagged Hdmx
c-Abl phosphorylates Hdmx and regulates its interaction with p53 proteins: wt, Y99F, Y55F, and Y55F,Y99F. To eliminate the effect of endogenous Hdmx on p53 interaction, endogenous Hdmx was downregulated by short hairpin RNA targeting the 3’-untranslated region (data not shown). Using this set of cell lines we examined the interaction between equivalent levels of Hdmx proteins and endogenous p53. p53 was immunoprecipitated (FL-393 antibody), and the amount of bound Hdmx protein was monitored with anti-HA antibody. Our results (Fig. 5C) revealed a slightly enhanced interaction between p53 and Y99F mutant, which is consistent with the above results (panel A). Interestingly, the interaction between p53 and Y55F and especially Y55F,Y99F mutants was reduced. These results strengthen the impact of these phosphorylation sites on the interaction between Hdmx and p53 (see “Discussion”).

FIGURE 6. Structural model; phosphorylation of Hdmx on Tyr-99 is expected to strongly interfere with its binding to p53. A, structure of the Mdmx protein (humanized zebrafish Mdmx; shown as a ribbon) bound to the p53 peptide (residues 15-37, shown as a stick model). Tyr-96 in the zebrafish protein is equivalent to Tyr-99 in human Mdmx. B, same structure as in panel A but with modelled phosphotyrosine (green). C, a close-up view of the interaction region with the van der Waals surface added. D, same as in panel C but with modelled phosphotyrosine (green).
Chapter 3

Effect of Stress on Hdmx Phosphorylations by c-Abl

Because c-Abl is activated upon stress, we examined the effect of stress conditions on Tyr-99 phosphorylation as a major c-Abl target site. HEK293 cells were transfected with expression plasmids for wt or Y55F,Y99F hdmx mutant, either alone or in combination with wt c-Abl or KD c-Abl. Cells were either left untreated or treated with a combination of H$_2$O$_2$ with tyrosine phosphatase inhibitor, sodium orthovanadate (Na$_3$VO$_4$), to activate c-Abl and prevent Hdmx dephosphorylation, respectively (36). Twenty-seven hours post-transfection Hdmx was immunoprecipitated, and protein complexes were analyzed by immunoblotting using anti-phospho-Tyr-99 Hdmx antibody. As shown in Fig. 7A, c-Abl-induced phosphorylation of Tyr-99 increased in response to these stress conditions. The H$_2$O$_2$/Na$_3$VO$_4$ treatment also induced a shift in the electrophoretic mobility of Hdmx. The reason for this shift is not clear, but it has been reported earlier that the DNA damage-induced mobility shift is dependent on phosphorylation of serine 403 (e.g. Ref. 29). To corroborate that the observed stress-induced Hdmx phosphorylation is not cell type-specific, we confirmed these results in p53/mdm2 double KO mouse fibroblasts (2KO 174.2) and in MCF-7 breast cancer cells (supplemental Fig. S4). Furthermore, to examine whether endogenous c-Abl can mediate Hdmx Tyr-99 phosphorylation, HEK293-T cells were transfected with expression plasmid for hdmx. Cells were either left untreated or treated with H$_2$O$_2$/Na$_3$VO$_4$ before being subjected to a phosphorylation assay. As shown in Fig. 7B, Tyr-99 phosphorylation was detected even in the absence of transfected c-Abl but only after H$_2$O$_2$/Na$_3$VO$_4$ treatment to activate endogenous c-Abl. Of note, this phosphorylation was not detected in cells expressing a kinase-defective mutant of c-Abl (Fig. 7A), which is due to the dominant negative effect of this mutant over endogenous c-Abl. The detection of endogenous Hdmx phosphorylation by endogenous c-Abl was unsuccessful, primarily due to the low expression levels of Hdmx after exposure to stress. Our findings of a potential dependence of Tyr-55 phosphorylations on Tyr-99 together with the differential effect of these phosphorylation sites on p53 interaction raised the possibility that these two phosphorylation sites may be regulated differently. To test this possibility, HEK293 cells were infected with c-Abl-GFP expressing lentivirus and then transfected with hdmx p53 BD expression plasmid. Cells were exposed at different time points to the genotoxic agent doxorubicin for 1 h, washed, and harvested at the same time (Fig. 7C, upper panel). As shown in Fig. 7C, phosphorylations of both Tyr-55 and Tyr-99 increased after genotoxic stress, with a slightly earlier onset for Tyr-99 phosphorylation. Notably, with time after exposure to stress, the increase in phosphorylation of Tyr-55 was more intense relative to the increase in Tyr-99 phosphorylation. These results show that Tyr-55 and Tyr-99 phosphorylations are elevated by DNA damage, but the kinetics of their regulation may differ.
c-Abl phosphorylates Hdmx and regulates its interaction with p53

**FIGURE 7. Stress induces Hdmx phosphorylations by c-Abl.** HEK293 (A) or HEK293-T cells (B) were transfected with the indicated plasmids. Cells were either left untreated or were treated with 3 mm H$_2$O$_2$ together with tyrosine phosphatase inhibitor sodium orthovanadate (Na$_3$VO$_4$, 1 mm) for 20 min before harvest. Twenty-four hours post-transfection Hdmx was immunoprecipitated from cell extracts using anti-Hdmx antibody. 6B1A antibody was used in A, and a mixture of rabbit polyclonal anti-Hdmx antibodies (p55, p56, and 1328) was used in B. Hdmx phosphorylated on Tyr-99 was detected with anti-phospho-Tyr-99 antibody, and total Hdmx level was monitored with 6B1A (A) or MX-82 (B) anti-Hdmx antibody. C, HEK293 cells were infected with c-Abl-GFP lentivirus. Seventy-two hours later cells were transfected with His and Myc-tagged hdmx p53 BD plasmid. Forty-eight hours post-transfection, cells were either left untreated or were treated at different time points with doxorubicin 2.5 μg/ml for 1 h. All the cells were harvested at the same time point. The time course of the experiment is shown in the upper panel. Hdmx p53 BD was isolated from cell extracts as described in Fig. 2C, and the protein complex was subjected to Western blotting using either anti-phospho-Tyr-55 antibody or anti-phospho-Tyr-99 antibody. Total Hdmx levels were monitored by blotting with anti-Hdmx antibody (MX-82). Stress-induced activation of p53 was followed by using anti-phospho-Ser20 antibody.
Chapter 3

DISCUSSION

p53 protein levels and activity are tightly regulated under normal and stress conditions. The negative regulation of p53 is governed largely by the Mdm proteins (for review, see Ref. 37). Deregulation of either Mdm proteins can be sufficient to suppress p53, as seen in 10–20% of human cancer cases bearing wt p53 (6). In normal cells the release of p53 from the inhibitory effects of the Mdm proteins is essential for the proper accumulation and activation of p53 (17). This relief involves a spectrum of posttranslational modifications of p53 and the Mdm2 proteins (for review, see Refs. 38 and 39). Additionally, recent studies show that Mdmx is also subjected to a spectrum of posttranslational modifications (for review, see Ref. 2). These include basal phosphorylation of Mdmx by CDK2/Cdc2(p34) proposed to regulate Mdm2 localization (40) and phosphorylation by CK1-α stimulating the Mdmx-p53 interaction (41). Additionally, Akt1 mediates Hdmx phosphorylation, leading to Hdmx stabilization and induced 14-3-3 binding (42). Upon stress, Mdmx is modified by key regulators of biological response to DNA damage like checkpoint kinases 1 and 2 (Chk1, Chk2) and ATM (for review, see Refs. 2 and 43). Furthermore, ATM-dependent phosphorylations of Mdmx are required for the efficient damage-induced Mdmx degradation, at least partially by affecting binding to herpes virus-associated ubiquitin-specific protease (HAUSP) (44), and also by stimulating the interaction with 14-3-3 proteins, which contributes to nuclear accumulation and degradation of Hdmx under DNA-damage conditions (29, 31). The degradation of Hdmx in response to stress is critical for p53 activation (for review, see Ref. 17). An additional important arm of the ATM stress response is the activation of c-Abl. We have previously shown that p53 activation by c-Abl requires the neutralization of the inhibitory effects of Mdm2 (22, 23). c-Abl interacts with and phosphorylates Hdm2, thereby impairing its E3 ligase activity toward p53 (24, 25). In addition, c-Abl stabilizes the interaction of p53 tetramer with certain promoters, such as p21 (45, 46).

In this study we explored a new pathway by which c-Abl regulates p53. We found that c-Abl interacts with Hdmx and phosphorylates it in cultured cells. Specifically, we identified Tyr-55 and Tyr-99 as c-Abl phosphorylation sites. These phosphorylations are independent of Hdmx phosphorylation by ATM or Chk2, and they do not affect 14-3-3/Hdmx binding. Importantly, however, both tyrosines reside within the p53 binding region. We found that phosphorylation of Tyr-99 inhibits the interaction between Hdmx and p53 (Fig. 5A). This finding is highly supported by a recent study describing the crystal structure of the N-terminus of Mdmx bound to a p53 peptide. This study highlighted Y96 (humanized zebrafish Mdmx equivalent to Tyr-99) as a critical residue for p53 binding (35). The addition of phosphorylated Tyr-99 to the modelled structure reveals the expected inhibition of p53 binding (Fig. 6). Because a physical interaction is required for the inhibition of p53
c-Abl phosphorylates Hdmx and regulates its interaction with p53

by Hdmx (47), interference with this interaction provides an efficient mechanism by which to modulate the Hdmx-p53 regulation. Our results imply that in response to stress, c-Abl protects p53 also by modulating the Hdmx-p53 interaction through Tyr-99 phosphorylation. This defines the first phosphorylation of Hdmx, which inhibits its interaction with p53. Consequently, it is predicted that this phosphorylation would impair the inhibitory effect of Hdmx on p53. To test this prediction we measured the effect of Y99F substitution on the inhibitory effect of Hdmx on p53 transcriptional activity using a luciferase reporter assay. Although the substitution mutant consistently inhibited more efficiently than wt Hdmx, the effect was very modest (supplemental Fig. S5). Why is this effect so small? First, we assume that only a fraction of Hdmx molecules undergo tyrosine phosphorylation, to which the phosphorylation mutant is compared. Second, it needs to be stressed that in this type of experiments c-Abl is an efficient activator of p53 (22–24). It is, therefore, difficult to achieve an optimal stoichiometry between Hdmx, c-Abl, and p53 that distinguishes the direct effect of c-Abl on p53 from the indirect effect via the Mdm proteins.

The phosphorylation of Hdmx on Tyr-55 did not impair its interaction with p53, and in some experiments it even seemed to enhance this interaction (supplemental Fig. S3). Moreover, interaction of endogenous p53 with Y55F and especially with Y55F,Y99F Hdmx was reduced as compared with wt and Y99F Hdmx (Fig. 5C), emphasizing the role of Tyr-55 in the p53-Hdmx interaction. Thus, c-Abl phosphorylates two tyrosines within the p53 binding region of Hdmx, which differently affect its interaction with p53. How could this observation be reconciled? In an attempt to understand this observation, we monitored the pattern of these phosphorylations in response to DNA damage, a stress signal known to activate c-Abl and p53. Although the phosphorylation of both sites was enhanced in response to DNA damage, the pattern of phosphorylation of the two sites differed. The extent of Tyr-99 phosphorylation increased moderately, with a possible temporary reduction at around 8–9 h after damage. On the other hand, Tyr-55 phosphorylation appeared with a short delay after Tyr-99 phosphorylation but continued to increase stronger with time after exposure (Fig. 7C and data not shown). Furthermore, Tyr-55 phosphorylation was impaired in the absence of Tyr-99, suggesting a potential sequential phosphorylation where Tyr-99 precedes Tyr-55 (Fig. 4). On the basis of these findings we propose a scenario whereby Tyr-99 phosphorylation occurs early after exposure to DNA damage, preventing Hdmx-p53 interaction, thereby contributing to p53 activation. Subsequent phosphorylation of Hdmx on Tyr-55 may counteract the effect of Tyr-99 on p53 interaction, thereby contributing to the recovery of p53 from stress-induced activation. Overall, we show here that c-Abl not only targets Hdm2, but also targets Hdmx, which contributes to its role in p53 regulation.
Acknowledgments

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c-Abl phosphorylates Hdmx and regulates its interaction with p53

References

Supplementary Figures

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Supplementary Figure S1: Hdmx N- and C-terminal deletion mutants interact with c-Abl in vivo. HEK 293 cells were transfected with indicated plasmids. Forty-eight hours post-transfection cell extracts were subjected to immunoprecipitation (IP) using anti-c-Abl K-12 polyclonal antibody. IP was followed by immunoblotting (IB) with anti-Hdmx MX-82 monoclonal antibody (A) or anti-Hdmx 6B1A monoclonal antibody (B). c-Abl levels were monitored by blotting with anti-c-Abl monoclonal antibody (ABL-148). Schematic representation of the Hdmx deletion mutants used in this study is presented in Fig. 1B. Dividing lines in A and B indicate that lanes were grouped from different parts of the same gel.
Supplementary Figure S2: Phosphorylation of Hdmx Y99 is not necessary for phosphorylation of Hdmx S367 or for binding of 14-3-3 to Hdmx. (A) HEK293-T cells were transfected with expression vectors for hdmx or hdmx-Y99. Cells were treated with 3 mM H₂O₂ for 20 min, together with tyrosine phosphatase inhibitor sodium orthovanadate (Na₃VO₄; 1 mM) for 4 hrs, either with or without treatment with 20 6M protease inhibitor MG132. Twenty-eight hrs post-transfection cells were harvested and extracts were analysed by Western blotting using anti-phospho-S367-Hdmx antibody. Total Hdmx levels were monitored by incubating with anti-Hdmx antibody (BL1258). (B) MCF7 cells were transfected with expression vectors for hdmx, hdmx-Y55F, hdmx-Y99F or hdmx-S367A. Cells were either non-treated or treated with Neocarzinostatin (NCS) (500 ng/ml) for 5 hrs before harvesting. Twenty-nine hours post-transfection cell extracts were subjected to immunoprecipitation (IP) using a mixture of rabbit anti-Hdmx antibodies p55, p56 and 1328. Immunoprecipitates were analysed by immunoblotting (IB) with anti-14-3-3tau antibody or anti-Hdmx antibody. Hdmx and 14-3-3tau levels in total extracts were investigated by immunoblotting with anti-Hdmx (BL1258) antibody and anti-14-3-3tau antibody, respectively.
c-Abl phosphorylates Hdmx and regulates its interaction with p53

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Supplementary Figure S3: c-Abl-dependent phosphorylation of Hdmx on Y55 enhances its binding to p53. H1299 cells were transfected with expression plasmids for hdmx p53 BD wt or Y55.99F phosphorylation mutant either alone or in combination with p53 and c-abl. Twenty-four hours later cell extracts were subjected to immunoprecipitation analysis using anti-p53 393-Ac antibody. The level of Hdmx was monitored with anti-Hdmx 1328 antibody. The extent of phosphorylated Y55 was monitored by blotting with anti-phospho-Y55 Hdmx antibody. The very same exposures are presented for the input and immunoprecipitated fractions which were run on the same gel.

Supplementary Figure S4: Hdmx is phosphorylated upon stress by c-Abl on Tyr-99 in different cell types. 2KO 174.2 (mdm2/p53 double knock-out MEFs) and MCF7 cells were transfected with expression plasmids for hdmx with or without c-abl. Cells were either non–treated or treated with 3 mM H2O2 together with tyrosine phosphatase inhibitor sodium orthovanadate (Na3VO4; 1 mM) for 20 min before harvest. Twenty-four hrs post-transfection Hdmx was immunoprecipitated from cell extracts using a mixture of rabbit polyclonal anti-Hdmx antibodies (p55, p56 and 1328). Hdmx phosphorylated on Y99 was detected with anti-phospho-Y99 antibody and total Hdmx level was monitored with anti-Hdmx antibody (MX-82).
Supplementary Figure S5: c-Abl-dependent phosphorylation of Hdmx at Y99 slightly inhibits Hdmx ability to inhibit p53. H1299 cells were transfected with the p53-reporter plasmid (Cyclin G-luc) either alone or in combination with p53 and c-abl with or without addition of wt or mutant hdmx p53 BD. Twenty-six hours later cell extracts were subjected to luciferase assay using Promega luciferase kit. Cells were treated with tyrosine phosphatase inhibitor sodium orthovanadate (Na$_3$VO$_4$; 0.3 mM) for 4.5 hours before harvesting.
Chapter 4

Oncogenic functions of hMDMX in \textit{in vitro} transformation of primary human fibroblasts and embryonic retinoblasts

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Chapter 4

Background
In around 50% of all human cancers the tumor suppressor p53 is mutated. It is generally assumed that in the remaining tumors the wild-type p53 protein is functionally impaired. The two main inhibitors of p53, hMDM2 (MDM2) and hMDMX (MDMX/MDM4) are frequently overexpressed in wild-type p53 tumors. Whereas the main activity of hMDM2 is to degrade p53 protein, its close homolog hMDMX does not degrade p53, but it represses its transcriptional activity. Here we study the role of hMDMX in the neoplastic transformation of human fibroblasts and embryonic retinoblasts, since a high number of retinoblastomas contain elevated hMDMX levels.

Methods
We made use of an in vitro transformation model using a retroviral system of RNA interference and gene overexpression in primary human fibroblasts and embryonic retinoblasts. Consecutive knockdown of RB and p53, overexpression of SV40-small t, oncogenic HRasV12 and HA-hMDMX resulted in a number of stable cell lines representing different stages of the transformation process, enabling a comparison between loss of p53 and hMDMX overexpression. The cell lines were tested in various assays to assess their oncogenic potential.

Results
Both p53-knockdown and hMDMX overexpression accelerated proliferation and prevented growth suppression induced by introduction of oncogenic Ras, which was required for anchorage-independent growth and the ability to form tumors in vivo. Furthermore, we found that hMDMX overexpression represses basal p53 activity to some extent. Transformed fibroblasts with very high levels of hMDMX became largely resistant to the p53 reactivating drug Nutlin-3. The Nutlin-3 response of hMDMX transformed retinoblasts was intact and resembled that of retinoblastoma cell lines.

Conclusions
Our studies show that hMDMX has the essential properties of an oncogene. Its constitutive expression contributes to the oncogenic phenotype of transformed human cells. Its main function appears to be p53 inactivation. Therefore, developing new drugs targeting hMDMX is a valid approach to obtain new treatments for a subset of human tumors expressing wild-type p53.
Oncogenic functions of hMDMX in \textit{in vitro} transformation

**Background**

In approximately 50\% of all human cancers mutations are found in the \textit{TP53} gene, encoding the tumor suppressor protein p53 \cite{1, 2}, whereas it is assumed that in tumors expressing wild-type p53 the tumor suppressing activity of p53 is attenuated \cite{3}. Normal, non-stressed cells maintain relatively low p53 protein levels. Upon various stress signals like DNA damage or oncogenic stress, p53 is stabilized and activated. Activated p53 affects various processes, including cell cycle progression, DNA repair, senescence and apoptosis \cite{4}. Two main negative regulators of p53 are MDM2 and MDMX, also called hMDM2 and hMDMX. MDM2, an E3 ubiquitin ligase, inhibits p53 via poly-ubiquitination \cite{5} and by binding to p53’s N-terminus, thereby shielding its transcription activation domain. Since the \textit{MDM2} gene is also a p53 target, a negative feedback-loop is established \cite{6}. The importance of MDM2 in p53 regulation was best shown by the p53-dependent embryonic lethality of \textit{MDM2 \textendash} mice \cite{7, 8}. Similarly, \textit{MDMX \textendash} mice are embryonic lethal in a p53-dependent manner \cite{9-11}, indicating that both MDM2 and MDMX fulfil an essential, non-redundant function in p53-regulation. Despite great structural similarities between MDM2 and MDMX \cite{12}, including the RING finger domain needed for MDM2 E3 ligase activity, MDMX has no detectable E3 ligase activity. MDMX functions mostly by inhibiting p53 activity through interaction with its transcription activation domain \cite{13, 14}. Furthermore, MDMX and MDM2 dimerize via their RING finger domains \cite{15}, thereby stabilizing MDM2 and promoting its E3 ligase activity towards p53 \cite{16, 17}.

hMDM2 is overexpressed in 5-10\% of all human tumors, revealing hMDM2 as an oncogene \cite{18}. Similar observations were made regarding hMDMX. A study of common tumor types showed increased \textit{hMDMX} mRNA expression in 20\% of these tumors \cite{19}, and a subset of gliomas contained \textit{hMDMX} gene amplification \cite{20}. Furthermore, Ramos \textit{et al.} showed upregulated or aberrant hMDMX expression in a large number of human tumor cell lines, mostly correlating with wild-type p53 status \cite{21}. A particularly high proportion of retinoblastomas contain \textit{hMDMX} gene amplification \cite{22}. hMDMX knockdown in p53 wild-type tumor cells has been shown to induce p53-dependent growth inhibition \cite{19, 22}.

The first evidence for direct oncogenic activity of MDMX was provided by Danovi \textit{et al.} \cite{19}. MDMX overexpression in early cultures of mouse embryonic fibroblasts resulted in immortalization and neoplastic transformation when combined with HRasV12 overexpression. This suggests that MDMX overexpression is sufficient to inactivate the p53 tumor suppressor pathway. However, such an oncogene function of hMDMX has not yet been directly shown in human cells.
Chapter 4

Human primary cells require a specific set of genetic changes for neoplastic transformation. By expression of the human Telomerase reverse transcriptase subunit (hTERT), oncogenic HRasV12, and the early region of SV40, encoding the viral large and small T antigens (LT and st), primary human cells can be immortalized and transformed. LT is needed to inactivate RB and p53, since functional loss of both genes is required for tumor formation [23, 24]. By combining this transformation model with specific RNA interference, the tumor-suppressive functions of p14ARF and p16INK4A were assessed by Voorhoeve and Agami [25]. They also showed that directly targeting p53 and RB could replace LT expression. Here we use a retroviral system of RNA interference and gene overexpression to establish an in vitro transformation model for assessing the contribution of hMDMX to the transformation of human primary cells.

Results

Generation of transformed human skin fibroblasts and human embryonic retinoblasts, including hMDMX as a potential oncogene

To investigate whether hMDMX can function as an oncogene in the transformation of human primary cells, we applied a previously described in vitro transformation model [25] to two different cell types: human foreskin fibroblasts (VH10) and human embryonic retinoblasts (HER).

The generation of the VH10 transformation model is depicted in Fig. 1A. Sequential retroviral transductions resulted in a panel of stable polyclonal cell lines, representing different stages of the transformation process. This enabled a pair-wise comparison between hMDMX overexpression and p53-knockdown. Stable cell lines with shRB-HA-hMDMX, or shRB alone, could not be established, suggesting that RB reduction is growth limiting in these cells. Oncogenic HRasV12 without concomitant p53-knockdown or HA-hMDMX overexpression induced a senescent-like crisis blocking proliferation of most cells, followed by expansion of single colonies. RB knockdown was not sufficient to rescue HRasV12-induced growth inhibition. Therefore, the cell lines VH10-shRB-HRasV12 and VH10-HRasV12 could not be established. We monitored the effectiveness of the transductions by western blotting (Fig. 1B) and found strong overexpression of hMDMX, somewhat increased HRas levels and marked reductions of p53 and RB, correlating with the respective transductions. Interestingly, endogenous HRas level was slightly increased upon p53-knockdown or hMDMX overexpression, correlating with
Oncogenic functions of hMDMX in \textit{in vitro} transformation

accelerated growth. HRasV12 has been reported to induce p16-dependent senescence in human fibroblasts [4, 25, 26]. Indeed, HRasV12-transformed as well as p53-knockdown cells expressed higher p16 protein levels (Fig.1B), although we observed no signs of senescence. This suggests that during the transformation process the pathway downstream of p16 is somehow impaired. Alternatively, p53 inactivation may prevent HRasV12-induced senescence, which was indeed described for hMDMX overexpression [27].

For HER transformation, we initially used a comparable approach. However, HRasV12-transformed cells could not be established without p53-knockdown or HA-hMDMX overexpression, which prompted us to modify the scheme (Fig. 1C). Transformation of HER-hTERT-shRB or HER-hTERT cells with an empty puromycin vector and subsequent puromycin selection resulted in initial colony formation, but these colonies eventually stopped growing. This suggests that in HER cells, in contrast to VH10, inactivation of p53 is essential for establishing immortalized cell lines. Protein levels of RB, hMDMX, p53 and HRas correlated with the applied transductions (Fig. 1D), although the RB depletion was less efficient in HA-hMDMX cells (lane 4 and 5). Similar to the observations in VH10 cells, HRasV12 expression induced p16 protein levels in HER cells. This did not affect growth of RB-knockdown cells, whereas HER cells without RB-knockdown eventually stopped proliferating upon HRasV12 overexpression. Likely, HRasV12 activated a p16- and RB-dependent mechanism resulting in growth suppression (also illustrated by large, flattened cells, Fig. 2C), which could not be rescued by p53-knockdown or hMDMX overexpression. Therefore, these cells could not be used in further experiments.

hMDMX overexpression and p53-knockdown were analyzed with qRT-PCR, showing approximately 90% reduction of \textit{p53} and 10-fold increase of \textit{hMDMX} mRNA expression in VH10 cells (Table 1 and not shown). Expression of p53 target genes \textit{p21}, \textit{PUMA} and \textit{hMDM2-p2} was significantly decreased upon p53-knockdown in VH10 and HER cells, and also hMDMX overexpression slightly decreased basal levels of some of these genes (Table 1 and Fig. 5B). Reduced \textit{p21} protein levels upon p53-knockdown (Fig. 1B and 1D), in line with the qRT-PCR data, indicate impaired basal p53 activity. hMDMX overexpression did slightly reduce basal \textit{p21} protein levels in VH10 cells (Table 2). In addition, in HA-hMDMX cells the protein levels of p53 were slightly increased, most likely by protein stabilization.
Figure 1. Generation of panels of transformed human skin fibroblasts and human embryonic retinoblasts.

A. Schematic representation of transformation process. Primary human fibroblasts (VH10) were immortalized with human Telomerase (hTERT). In subsequent rounds of retroviral infection using the indicated constructs, followed by selection, several stable cell lines were created. B. Total cell extracts from all transformed fibroblast cell lines were analyzed by immunoblotting with the indicated antibodies. C. Human Embryonic Retinoblasts (HER) were similarly transformed according to the scheme. D. Total cell extracts from all transformed HER cell lines were analyzed by immunoblotting with the indicated antibodies.
Oncogenic functions of hMDMX in *in vitro* transformation

Table 1. mRNA expression levels in VH10 and HER cell lines

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<td>0.20 ± 0.03</td>
<td>0.49 ± 0.11</td>
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<tr>
<td>HRasV12-shp53</td>
<td>0.15 ± 0.03</td>
<td>0.32 ± 0.07</td>
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<td>0.63 ± 0.11</td>
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<tr>
<td>HA-hMDMX</td>
<td>0.47 ± 0.09</td>
<td>0.61 ± 0.08</td>
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<td>2.52 ± 0.70</td>
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Total RNA of each cell line was isolated and expression levels of the indicated genes were determined by qRT-PCR and normalized for the housekeeping genes *CAPNS1* and *TBP*; levels are shown relative to wild-type cells.

Table 2. Protein levels, relative to untreated VH10 wt cells, corrected for HAUSP expression.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Nutlin-3 treatment (h)</th>
<th>p53</th>
<th>p21</th>
<th>hMDM2</th>
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<td>1.00</td>
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<td>2.26</td>
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<td>1.76</td>
<td>4.28</td>
<td>79.91</td>
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Band intensities shown in figure 3B were quantified using the Odyssey 2.1 analysis software (LI-COR Biosciences) and the relative protein levels were calculated using HAUSP expression as an internal control. Basal protein levels in VH10 wild-type cells were set at 1.0.
Immunofluorescence analysis revealed abundant GFP throughout the entire cell in all established HER and VH10 cell lines, which confirmed SV40-st expression (Add. File 1A and 1C).

Exogenous hMDMX showed mainly cytoplasmic localisation in VH10 cells, but nuclear hMDMX was also observed (Add. File 1A). In HER cells, the main localization was nuclear (Add. File 1B). High levels of cytoplasmic hMDMX were reported to prevent p53 nuclear localization [28]. However, we found no alterations in p53 localization upon hMDMX overexpression (Add. File 1B and 1D). hMDM2 protein, irrespective of hMDMX levels, was detected in the nucleus, although it could be observed in the cytoplasm as well. The cytoplasmic signal of hMDM2 is relatively underrepresented since the protein is diffused throughout the relatively large cytoplasmic surface, but is certainly present, as reported before [29] (Add. File 1B and D).

Since p53 contributes to the maintenance of genomic stability [4], the various cell lines were analyzed for chromosomal abnormalities using COBRA-FISH [30]. Wild-type VH10hTERT cells (Add. File 2A) showed a normal 46,XY karyogram in 20% of the analyzed cells. The remaining cells harboured a Robertsonian translocation [31], which results in loss of the short arms of two acrocentric chromosomes. As these contain the ribosomal gene cassettes, this translocation has no further consequences at the cellular level. The observed rob(13;22) chromosome was lost during the transformation process. The transformed VH10 cell lines showed heterogeneous populations of mainly diploid and chromosomal stable cells, with low percentage random translocations or polyploidy. The HER cells showed more chromosomal aberrations and translocations (Add. File 2B). Most notably, loss of chromosome 13, harbouring the RB gene, was found in four out of six cell lines. The fact that it was not found in the HA-hMDMX and shRB-HA-hMDMX-HRasV12 cell line suggests that loss of this chromosome occurred independently in those four cell lines (see Fig. 1C for transformation scheme). Two unlinked cell lines (shp53 and shRB-HA-hMDMX-HRasV12) lost one X-chromosome, whereas loss of chromosome 22 and gain of chromosome 8 in shRB-shp53 and shRB-shp53-HRasV12 is likely to have been passed on from their shared parental cell line. In addition, several random translocations and fusions were observed, however, none was found in more than one cell line. In conclusion, transformation of VH10 and HER cells did not induce wide-spread genomic instability and aneuploidy.
Alterations in morphology and proliferation rate during transformation process

Cell morphology changed during the transformation process. Whereas normal VH10hTERT cells are extended, fibroblastic cells aligning orderly in the dish, during the sequential transformation stages the cells became apparently smaller and rounder (Fig. 2A). HrasV12 induced a disordered way of growing and showed loss of contact inhibition. hMDMX-overexpressing and p53-knockdown cells showed similar morphology changes. Both shp53- and HA-hMDMX cells obviously proliferated faster than wild-type VH10hTERT cells, a property that was not further enhanced by additional HrasV12 expression. This was confirmed in short-term growth (WST-1) assays (Fig. 2B), suggesting that hMDMX overexpression is sufficient to inhibit p53-dependent growth control, similar to p53-knockdown.

Morphology changes in transformed HER cells were comparable to those observed in VH10 cells (Fig. 2C). In addition, both p53-knockdown and hMDMX overexpression accelerated growth as compared to wild-type HER cells (Fig. 2D). HrasV12 even further enhanced growth rate in shRB-shp53 cells, but not in shRB-HA-hMDMX cells.

Effect of hMDMX overexpression on anchorage-independent growth

Anchorage-independent growth is a vital feature of tumorigenic cells. Therefore, we investigated the growth potential of the different cell lines in soft agar (Fig. 3A). Control VH10hTERT cells did not grow, but both p53-knockdown and hMDMX overexpression induced formation of some small colonies. HrasV12 expression clearly increased the size and the number of colonies. However, this was more pronounced in p53-knockdown cells than in hMDMX-overexpressing cells. Interestingly, hMDMX overexpression did not prevent the growth inhibitory effect of Nutlin-3, in contrast to experiments in 2D-culture (see below). These findings suggest that hMDMX cannot fully inhibit the function of p53 in soft agar growth.

Untransformed HER cells did not grow at all in soft agar, and neither hMDMX overexpression nor p53-knockdown alone was able to induce colony formation (not shown). Although additional RB knockdown induced formation of very small colonies, only HrasV12 expression dramatically increased colony size and number (Fig. 3B). Similar to the observations in VH10 cells, colony formation was more efficient in p53-knockdown cells than in hMDMX-overexpressing cells. Furthermore, Nutlin-3 inhibited colony formation of hMDMX-overexpressing cells, whereas p53-knockdown cells were unaffected.
Figure 2. Transformation alters cell morphology and growth rate.
Phase-contrast photographs of the transformed VH10 (A) and HER (C) cell lines (10x magnification, Olympus CKX41) showing morphology changes during the transformation process. Growth rates of VH10 (B) and HER (D) cell lines were measured using WST-1 proliferation assays.
Oncogenic functions of hMDMX in *in vitro* transformation

Figure 3. hMDMX overexpression promotes anchorage-independent growth and tumor growth *in vivo*. A, B. Various VH10hTERT and HER cell lines were embedded in 0.3 % agarose on a 0.6% agarose bottom-layer, with additional normal growth medium or growth medium containing 10 µM Nutlin-3 on top of the agarose. Colony outgrowth was monitored and pictures were taken 18 days (VH10) and 4 weeks (HER) after seeding. Representative pictures of several independent experiments are shown. C. Schedule for investigating *in vivo* growth of parental and transformed VH10 cells using the shell-less chicken CAM model. At embryonic development day (EDD) 7, 2.5 million cells were grafted onto a chicken CAM. Tumors were harvested at EDD17. D. Representative pictures of GFP-positive tumors, P-Histone-3 staining and vimentin staining. E. Tumor volumes of VH10hTERT control (N=5), shRB-HRasV12-shp53 (N=4) and shRB-HRasV12-HA-hMDMX (N=5). F. Quantification of the number of mitotic (P-Histone-3 positive) cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s Multiple Comparison Test. P<0.05 was considered statistically significant.
Role of hMDMX in tumorigenicity in vivo.

We tested the *in vivo* tumorigenic potential of the transformed VH10 cells by subcutaneous injection into Balb/c nu/nu mice. Unfortunately, no tumor formation could be detected. The lack of tumor growth could possibly be explained by the immune response still present in nu/nu mice; however, similar results were obtained in NOD/SCID mice. Therefore, we switched to the shell-less chick CAM assay [32] (Fig. 3C-F). Ten days after grafting both shRB-HRasV12-HA-hMDMX- and shRB-HRasV12-shp53 tumors were significantly larger (4-5 fold) than those formed by wild-type VH10 cells (P < 0.05, Bonferroni’s Multiple Comparison Test; Fig. 3D left and E). Interestingly, the transformed cell lines showed equal tumor volumes, indicating that hMDMX overexpression and p53-knockdown have similar effects on tumor growth. Moreover, tumors from the transformed cells contained significantly (P<0.05) more mitotic cells than wild-type tumors as revealed with P-Histone-3 staining, with no detectable difference between hMDMX-overexpressing and p53-knockdown cells (Fig. 3D middle and 3F). Staining with fibroblast marker vimentin showed the fibroblastic origin of the tumors (Fig 3D right).

The *in vivo* growth capacity of transformed HER cells was tested in a previously described murine model [33], by injection into the anterior eye chamber of Balb/c nu/nu mice. Both tested cell lines showed similar, but limited *in vivo* growth potential. The hMDMX-expressing cells showed growth in 2/5 cases, but growth stopped when the eye chamber was filled up to 50% with tumor cells. The p53-knockdown cells started tumor growth in 4/5 cases, but stopped when the eye chamber was filled up to 20% (2x), 30% (1x) or 50% (1x). Altogether, it is clear that hMDMX overexpression promotes *in vivo* tumor growth and in that respect largely mimics p53-knockdown in the same cells.

**hMDMX overexpression inhibits the Nutlin-3 induced p53 response in VH10 skin fibroblasts.**

We next investigated whether hMDMX overexpression prevents p53 activation in VH10 cells. The various cell lines were treated with the small-molecule p53-activator Nutlin-3 [34]. Nutlin-3 reduced survival of normal human fibroblasts (Fig. 4A), whereas p53-knockdown cells were not affected. hMDMX overexpression also prevented Nutlin-3 induced growth inhibition. In wild-type VH10hTERT cells, the Nutlin-3 response is marked by increased p53, hMDM2 and p21 and decreased hMDMX protein levels (Fig. 4B). This response was diminished upon p53-knockdown, reaching levels slightly above basal expression in wild-type cells (Table 2). HA-hMDMX overexpression also attenuated the induction of p53 and its target genes. Notably, exogenous HA-hMDMX levels remained relatively high, despite some Nutlin-3 induced degradation. Levels of the p53-responsive
Figure 4. hMDMX overexpression inhibits Nutlin-3 mediated p53 activation in human fibroblasts.

A. Various VH10hTERT cell lines were continuously treated with 10 μM Nutlin-3 and proliferation was measured after 96 hours using a WST-1 assay. Relative cell numbers are displayed as survival relative to untreated cells. B. Various VH10hTERT cell lines were treated for the indicated times with 10 μM Nutlin-3, and protein levels were analyzed with immunoblotting using the indicated antibodies. C. qRT-PCR analysis of cells treated as in B. Expression levels of hMDM2-p2, p21, PUMA and SURVIVIN are shown as the fold induction relative to untreated wild-type VH10 cells.
transcripts of hMDM2-p2, p21 and PUMA correlated with protein levels (Fig. 4C). Furthermore, hMDMX overexpression partially rescued the reduction of the anti-apoptotic gene SURVIVIN by Nutlin-3 [35]. Table 3 shows fold changes of p53, hMDM2, hMDMX and the p53 targets hMDM2-p2, p21, PUMA, GADD45-alpha and SURVIVIN, for each cell line separately. As expected, p53 and hMDMX mRNA levels did not significantly change upon Nutlin-3 treatment.

hMDMX overexpression in HER cells is not sufficient to inhibit the Nutlin-3 induced p53 response.

Similar to the observations in VH10, in HER cells Nutlin-3 increased p53, hMDM2 and p21 and reduced hMDMX protein levels, which was efficiently blocked by p53-knockdown (Fig. 5A). Surprisingly however, hMDMX overexpression hardly rescued these effects. Although at the mRNA level (Fig. 5B) the inductions of p53 targets hMDM2-p2, p21 and PUMA were indeed slightly attenuated, this appeared to be insufficient to prevent Nutlin-3 induced growth inhibition, as illustrated by reduced survival (Fig. 5C) and S-phase depletion (Fig. 5D).

To explain the differences between the hMDMX-overexpressing VH10 and HER cells in their Nutlin-3 response, we compared protein and mRNA levels side-by-side (Fig. 5E and F). Strikingly, HA-hMDMX was clearly higher expressed in VH10 cells than in HER cells, while endogenous p53, hMDM2 and p21 levels were comparable. As Nutlin-3 not only binds hMDM2 but also hMDMX, albeit with much lower affinity [22], the levels of hMDMX may affect Nutlin-3 sensitivity. In HER cells, the remaining hMDMX levels after Nutlin-3 treatment may not be sufficient to prevent p53 activity. Importantly, we found that hMDMX levels in transformed HER cells were comparable to the levels in retinoblastoma cell lines Y79 and Weri1 (Fig. 5G). We have previously shown that in these retinoblastoma cells p53 is inhibited via high hMDMX expression, and that they are sensitive to Nutlin-3 [22]. These findings indicate that the transformed retinoblasts provide a representative model for retinoblastoma.

The response to Nutlin-3 may also be determined by E2F1 activity, which activates p73. Kitagawa et al. [36] reported that Nutlin-3-induced downregulation of E2F1 correlates with relative Nutlin-3 resistance, and they suggested that cells lacking RB activity are much more prone to entering Nutlin-3-induced apoptosis. Therefore, we compared RB and E2F1 levels in a selected panel of VH10 and HER cell lines (Fig. 5E). As reported before [37], Nutlin-3 decreased total and hyper-phosphorylated (upper band) RB. However, the amount of hypo-RB (lower band) was hardly affected. Moreover, after Nutlin-3 treatment, the
Figure 5. hMDMX overexpression in HER cells is not sufficient to prevent the Nutlin-3 induced p53-activation, which resembles retinoblastoma cell lines.

A. The various HER cell lines were treated with 10 µM Nutlin-3 for 24 hours, and protein levels were analyzed with immunoblotting using the indicated antibodies. B. qRT-PCR analysis of cells treated as in A. Expression
levels of hMDM2-p2, p21 and PUMA are shown as the fold induction relative to untreated wild-type HER cells. C. The various HER cell lines were continuously treated with 10 μM Nutlin-3 and proliferation was measured after 120 hours using a WST-1 assay. Relative cell numbers are displayed as survival relative to untreated cells. D. Cells were treated with 10 μM Nutlin-3 for 24 hours and analyzed by flow cytometry. Percentages of cells in S-phase are displayed as indicative for cell proliferation. E. Parental and shRB-HRasV12-HA-hMDMX (RRHX) transformed VH10 and HER cells were treated with 10 μM Nutlin-3 for 24 hours and analyzed with immunoblotting using the indicated antibodies. F. Quantification of the indicated protein levels using Odyssey 2.1 analysis software (LI-COR Biosciences) for at least two different exposures. Relative protein levels were calculated using Vinculin expression as an internal control and indicated as fold induction relative to untreated cells. G. Parental and RRHX transformed HER cells and the retinoblastoma cell lines Y79 and Weri1 were treated with 10 μM Nutlin-3 for 24 hours, and analyzed with immunoblotting using the indicated antibodies.

hypo-RB levels in normal and RB-knockdown cells were comparable, and the differences between VH10 and HER cells were rather small. E2F1 reduction at the protein (Fig. 5E) and mRNA level (Add. File 3A, upper panel) as observed in the parental VH10 and HER cells was attenuated in the transformed cells. The mRNA levels of the E2F1 target gene CDC25a followed a similar pattern. Nutlin-3 also reduced the expression of both total p73 (Add. File 3A, lower panel) and TA-p73 (not shown) in the parental cells. Strikingly, basal p73 expression was strongly reduced upon transformation. However, transformed VH10 and HER cells showed comparable E2F1 regulation and p73 expression, so this cannot explain the observed differences in Nutlin-3 sensitivity. Therefore, these dissimilarities are more likely the result of different HA-hMDMX levels.

hMDMX overexpression in VH10 cells also inhibited p53 activation by 5-FU and etoposide on both mRNA and protein level (Add. File 3B and C), similar as observed with Nutlin-3 treatments. However, reduced proliferation in response to these drugs occurred mainly through p53-independent pathways, because neither p53-knockdown nor hMDMX overexpression did not rescue the growth inhibitory effect (Add. File 3D). Nevertheless, FACS analysis showed different responses between wild-type, hMDMX-overexpressing and p53-knockdown VH10 cells (data not shown). Upon etoposide treatment, wild-type and hMDMX-overexpressing cells showed a two-fold reduction of G1 phase and an increased G2 fraction. The G2 arrest in p53-knockdown cells was much more severe, with less than 10% remaining in G1. 5-FU induced S-phase accumulation and G2 reduction in wild-type and HA-hMDMX expressing cells, whereas p53-knockdown cells strongly accumulated in G1.
Table 3. Fold induction mRNA per cell line after Nutlin-3 treatment.

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<tr>
<th>Cell line</th>
<th>Nutlin-3 treatment (h)</th>
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<th>p21</th>
<th>hMDM2</th>
<th>hMDM2-p2</th>
<th>hMDMX</th>
<th>PUMA</th>
<th>GADD45a</th>
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<td>1.00 ± 0.16</td>
<td>1.00 ± 0.17</td>
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<td>1.00 ± 0.09</td>
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<td>2.49 ± 0.67</td>
<td>1.68 ± 0.66</td>
<td>6.32 ± 0.79</td>
<td>1.18 ± 0.35</td>
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<td>1.00 ± 0.08</td>
<td>1.00 ± 0.34</td>
<td>1.00 ± 0.14</td>
<td>1.00 ± 0.69</td>
<td>1.00 ± 0.58</td>
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<td>3.47 ± 1.26</td>
<td>11.83 ± 5.00</td>
<td>1.00 ± 0.42</td>
<td>3.65 ± 0.35</td>
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<td>24</td>
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<td>6.86 ± 1.71</td>
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<td>13.6 ± 18.4</td>
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<td>26.21 ± 2.95</td>
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<td>0.19 ± 0.26</td>
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<td>10.36 ± 2.94</td>
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<td>0.14 ± 5.33</td>
<td>1.27 ± 1.71</td>
<td>0.28 ± 0.93</td>
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</table>

VH10 cells were treated with 10 µM Nutlin-3 for the indicated times and analyzed using qRT-PCR. Expression was normalized for the housekeeping genes CAPNS1 and TBP. The induction of mRNA expression upon Nutlin-3 treatment per cell line is shown for p53, p21, hMDM2, hMDM2-p2, hMDMX, PUMA, GADD45alpha and SURVIVIN. For each cell line the basal mRNA expression is set at 1.0.

Discussion

In this study, we analyzed the putative oncogenic function of hMDMX in the neoplastic transformation of normal human skin fibroblasts (VH10) and Human Embryonic Retinoblasts (HER). We chose retinoblasts since hMDMX is frequently overexpressed and/or amplified in retinoblastoma development. Retinoblastomas, like most other human tumors with increased hMDMX levels, retain wild-type p53 [19-22], suggesting that the oncogenic function of hMDMX is primarily based upon p53 inhibition. After RB inactivation, E2F1 is activated resulting in elevated p14ARF levels, repression of hMDM2 and activation of p53. Since hMDMX is not inhibited by p14ARF, hMDMX-overexpressing cells escape the p53-mediated cell death [38].

Indeed, we find that constitutive expression of hMDMX in foreskin fibroblasts functionally strongly resembles p53-knockdown cells. In combination with other defined genetic changes, hMDMX expression contributes to neoplastic transformation. In transformed cells, hMDMX overexpression reduces basal mRNA and protein levels of p53 targets, with exception of hMDM2 protein levels which are increased most likely via hMDMX-mediated stabilization. Vice versa, the expression of p53-repressed genes, like SURVIVIN, is increased. The ultimately obtained transformed cells show anchorage-independent growth, and can form tumors in an in vivo model.
Similarly, hMDMX-expressing HER cells largely resemble p53-knockdown HER cells regarding transformed properties, although hMDMX is less able to counteract the oncogenic HRas-induced growth inhibition, even in RB-knockdown cells. The ultimately obtained transformed cells, with either hMDMX overexpression or p53-knockdown, show \textit{in vivo} growth capacity, although limited.

Our results support the idea that the hMDMX overexpression, which is found in a subset of human tumors [19-22], is an important step in the development of that tumor, and that its main function is to inactivate p53. Interestingly, recently two transgenic mouse models have been described that widely overexpress MDMX [39, 40]. Surprisingly, the phenotypes were very different. Whereas mice from the Lozano lab spontaneously developed tumors upon MDMX overexpression [39], no spontaneous tumor formation nor cooperation with Eµ-Myc-induced tumors was observed in the mice from the Marine lab [40]. In both cases the MDMX-overexpressing MEFs or thymocytes showed an attenuated p53 response upon Nutlin-3 and IR treatment, respectively, suggesting the expression of a functional MDMX protein. It will be important to carefully examine these two mouse models to understand the distinct phenotype. This might teach us more about functions of MDMX in tumorigenesis.

In line with these studies, we find that hMDMX overexpression attenuates the Nutlin-3 mediated p53 activation and growth inhibition in skin fibroblasts. Nutlin-3 has a much lower affinity for hMDMX compared to hMDM2 [22, 41], so the effect of hMDMX overexpression is probably caused by direct p53 inhibition. Similarly, hMDMX overexpression reduces p53 activation by etoposide and 5-FU.

More strikingly, the hMDMX-overexpressing HER cells are still sensitive to Nutlin-3. The p53-response is hardly affected, both regarding regulation of p53 target genes and inhibition of cell proliferation. This difference with hMDMX-transformed VH10 cells is probably due to the lower hMDMX levels in HER-hMDMX cells, which are even further reduced by Nutlin-3. In that respect, the Nutlin-3 response of the transformed retinoblasts resembles that of retinoblastoma cell lines. As we have shown before, these retinoblastoma cell lines are still sensitive to Nutlin-3 and even show an apoptotic response, despite high levels of hMDMX [22].

High hMDMX expression has been reported to attenuate the Nutlin-3 response [42-44]. In a study by Patton and colleagues [43], human embryonic lung fibroblasts were transformed using hTERT, E1A, and oncogenic Ras, with either hMDMX or hMDM2 overexpression, or p53-knockdown, and Nutlin-3 sensitivity was assessed. They found that hMDMX overexpression, in contrast to hMDM2, prevented p53 activation upon Nutlin-3 treatment,
Oncogenic functions of hMDMX in in vitro transformation

which fits most of our data. Nutlin-3 did not inhibit soft agar growth of hMDMX-overexpressing cells in their study. By contrast, we found partial inhibition of soft agar growth by Nutlin-3, whereas growth in a monolayer was not affected at all. Possibly, in 3D additional stress is posed upon the cells, causing super-activation of p53 that cannot be completely counteracted by hMDMX proteins.

The discussed fibroblast models also show a different Nutlin-3 response: IMR-90 cells entered apoptosis, whereas in VH10 cells Nutlin-3 mainly inhibited cell growth without induction of apoptosis (data not shown). Notably, IMR-90 cells are embryonic lung cells; embryonic cells are less differentiated and can be more easily transformed. Furthermore, Patton et al. used adenovirus E1A for RB inactivation, but E1A proteins have additional growth affecting functions, including attenuation of the p53 response by interacting with p300/CBP [45, 46]. Therefore, a clean appreciation of the effects of hMDMX on the p53 response in the presence of E1A is difficult.

Beside hMDMX levels, also other factors may be involved in determining the outcome of Nutlin-3 treatment. Kitagawa et al. [36] have shown that RB status and E2F1 activity are important contributors. However, we found only minor changes in E2F1 activity in our model, which cannot explain the differences in Nutlin-3 sensitivity. Interestingly, the E2F1 target TA-p73 was dramatically decreased upon transformation. This might be a result of HRasV12 activity; oncogenic Ras has been described to switch the expression from TA-p73 to the antagonistic ΔN-p73, an important step during transformation. TA-p73 was reported to prevent anchorage-independent growth via activation of KCNK1 [47]. However, since transformed fibroblasts as well as retinoblasts express low levels of p73, this does not provide an explanation for the differential Nutlin-3 responses.

Conclusions

In conclusion, we find that hMDMX overexpression can replace loss of p53 during the transformation process of human fibroblasts and embryonic retinoblasts. In addition, very high hMDMX levels, as observed in VH10 cells, can prevent p53 activation by Nutlin-3. However, lower hMDMX levels like in the HER cells can no longer inhibit p53 after Nutlin-3 treatment, because hMDMX protein is mostly degraded by elevated hMDM2 levels, as previously shown in other tumor cells [48]. The Nutlin-3 response of the transformed HER cells resembles that of retinoblastoma cell lines, indicating that this is a physiologically relevant model. A combination therapy using Nutlin-3 and a specific hMDMX inhibitor, possibly a low dose of a DNA damaging agent leading to hMDMX degradation, might result in more effective treatment of tumors expressing wild-type p53 and high levels of hMDMX.
Table 4. List of used antibodies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name/ cat. #</th>
<th>Company</th>
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<tbody>
<tr>
<td>hMDMX</td>
<td>A300-287A</td>
<td>Bethyl Laboratories, Montgomery TX, USA</td>
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<tr>
<td>hMDMX</td>
<td>6B1A</td>
<td>[50]</td>
</tr>
<tr>
<td>HA-tag</td>
<td>HA.11</td>
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</tr>
<tr>
<td>HA-tag</td>
<td>ab9110</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>p53 °</td>
<td>DO-1 / sc-126</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>p53 °</td>
<td>PAb 1801 / sc-98</td>
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</tr>
<tr>
<td>p53</td>
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</tr>
<tr>
<td>hMDM2 * /MDM2</td>
<td>4B2</td>
<td>[51]</td>
</tr>
<tr>
<td>hMDM 2 *</td>
<td>SMP14 sc-6965</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>p21</td>
<td>CP74 / 05-655</td>
<td>Upstate Biotechnology, Lake Placid, NY, USA</td>
</tr>
<tr>
<td>RB</td>
<td>G3-245 / 554136</td>
<td>BD Pharmingen, Franklin Lakes, New Jersey, USA</td>
</tr>
<tr>
<td>HRas</td>
<td>Y13-259</td>
<td>[52]</td>
</tr>
<tr>
<td>HAUUSP/USP7</td>
<td>A300-033A</td>
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<tr>
<td>HAUUSP/USP7</td>
<td>7G9</td>
<td>[53]</td>
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<td>p21</td>
<td>CP74 / 05-655</td>
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<tr>
<td>γ-tubulin</td>
<td>GTU-88 / T6557</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
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</table>

Antibodies used in this study are listed in table 4, together with name or category number and the name of the company. For detection of human p53 we used a mix of DO-1 and 1801 (°), for detection of human Hdm2 we used a mix of 4B2 and SMP14 (*).

Table 5. Primer sequences

<table>
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<th>Sequence</th>
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<td>hMDM2-P2 Fw</td>
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<td>hMDM2-P2 Rv</td>
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<tr>
<td>P53 Fw</td>
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</tr>
<tr>
<td>P53 Rv</td>
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<tr>
<td>hMDMX Rv</td>
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<tr>
<td>p21 Rv</td>
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<tr>
<td>CAPNS1 Rv</td>
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<tr>
<td>CDC25A Rv</td>
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Sequences of qRT-PCR primers used in this study.
Oncogenic functions of hMDMX in *in vitro* transformation

**Methods**

**Generation of stably transformed human cell lines**
Primary human fibroblasts (VH10) and Human Embryonic Retinoblasts (HER) were immortalized by introducing human Telomerase (hTERT). Cells were maintained in DMEM supplemented with 10% FBS, 1% glutamine, antibiotics, amino-acids, glucose and vitamins. Stably transformed cell lines were generated in subsequent retroviral infection rounds according to the transformation schemes in Fig. 1A and 1C. pRetroSuper-shRB-Hygro and pRS-Hygro [25] were used for RB-knockdown or control cell lines, followed by hygromycin selection (50 µg/ml). pMSCV-blast-Ras or pMSCV-blast [25] were used for HRasV12 overexpression or control cell lines, followed by blasticidin selection (5 µg/ml). pBABE-HA-hMDMX-puro or pRS-shp53-puro [25] were used for hMDMX overexpression or p53-knockdown, both combined with pMSCV-GFP-st [25] for SV40-small-t expression, followed by puromycin selection (0.5 µg/ml). Cell lines were maintained under selection pressure.

**Immunoblotting**
Cells were lysed in Giordano 250 buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA), with protease- and phosphatase inhibitors. Proteins were separated by SDS-PAGE, transferred onto polyvinyl/dene difluoride membranes (Immobilon-P, Millipore) and incubated with the appropriate primary (listed in Table 4) and HRP-conjugated secondary antibody (Jackson Laboratories). Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce). Alternatively, membranes were incubated with secondary antibodies coupled to IRdye-680 and IRdye-800 near Infrared dyes (LI-COR Biosciences), and analyzed with the Odyssey Infrared Imager (LI-COR Biosciences).Signals were quantified using the Odyssey 2.1 analysis software (LI-COR Biosciences).

**Immunofluorescence**
Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2 % Triton X-100 for 10 min, blocked with 5% Normal Goat Serum (NGS) for 1 hour and incubated with primary antibodies for 1.5 hours and anti-mouse-Rhodamine secondary antibody (Jackson Laboratories) for 30 min. Coverslips were mounted onto microscope slides using DAPI-DABCO mounting solution.

**RNA isolation, qRT-PCR**
RNA was isolated using the SV Total RNA isolation kit (Promega, Madison, WI). cDNA was synthesized using 1.0 µg RNA in Reverse Transcriptase reaction mixture (Promega). Samples were analyzed in triplicate using SYBR Green mix (Roche Biochemicals, Indianapolis, IN) in a 7900ht Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For normalization the geometric mean of at least two housekeeping genes was used. Primer sequences are available in Table 5.

**Growth assay, soft agar assay**
For growth assays, 1000 cells were seeded in triplicate in 96-wells plates; treatments were started 24 hours after seeding. Cells were incubated with WST-1 reagent (Roche) for 1-4 hours and absorbance (450 nm) was measured in a microplate reader (Victor3 Multilabel Counter 1420-042, Perkin-Elmer).

Soft agar assays were performed in 96-well plates (VH10) or 6-well plates (HER) coated with a 0.6 % agarose bottom-layer. Per well, 5000 VH10 or 20,000 HER cells were seeded in 0.3 % agarose. Colony outgrowth was monitored (10x magnification, Olympus CKX41) and pictures were taken at several time points.

**Flow cytometry**
Cells were harvested, washed with PBS and fixed ice-cold 70 % ethanol. Cells were washed in PBS and incubated in PBS containing 50 µg/ml propidium iodide and 50 µg/ml RNase. Flow cytometry was performed in a BD LSR II system (BD Biosciences).

**Cytogenetic methods and combined binary ratio fluorescence in situ hybridization (COBRA-FISH)**
Culturing, harvest conditions and karyotyping were performed according to standard protocols [49]. Slides with metaphase chromosomes were hybridized using a multicolor FISH approach. Staining, digital imaging, and
Chapter 4

analysis were performed as described previously [30]. Hybridizations with individual libraries labeled with single fluorochromes were used to confirm the detected rearrangements. Chromosomal breakpoints were assigned by using inverted images counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Downers Grove, IL) together with the information derived from the short- and long-arm specific hybridization during COBRA-FISH. Karyotypes were described according to ISCN 2009.

Shell-less Chicken Chorioallantoic Membrane (CAM) assay

Fertilised chicken eggs were incubated at 37°C in humidified atmosphere. After 4 days they were cracked open into plastic dishes. At day 7, two-and-half million cells transduced with turbo-GFP lentiviral construct (SHC003, Sigma-Aldrich) were mixed with 50 µl basement membrane matrix (BD Biosciences) and grafted onto the CAM. At day 17, tumors with surrounding CAM were removed and the size was measured. GFP-positive tumors were photographed using a fluorescence stereomicroscope. Tumors were embedded in paraffin, sectioned and stained with anti-Vimentin, clone V9 (Santa Cruz) and anti-phospho-Histone-3 (Upstate, Millipore). Percentage of proliferating cells was calculated by quantifying phospho-Histone-3 positive nuclei of on average 500 nuclei from 5 random pictures per sample.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KLe and JdL performed the transformations of the human cells, performed all growth assays and contributed to the protein and mRNA analyses and immunofluorescence data. KLo and EW performed the in vivo tumorigenicity studies. AT, KLo and MVdV performed protein and mRNA analyses and contributed to the immunofluorescence data. MvdB and KS performed and interpreted the COBRA-FISH analyses. KLe, JdL and AGJ designed and coordinated the study and drafted the manuscript. All authors have read and approved the final manuscript.

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Oncogenic functions of hMDMX in *in vitro* transformation

References

Chapter 4


Oncogenic functions of hMDMX in *in vitro* transformation


Additional File 1. Overexpressed HA-hMDMX is localised both nuclear and cytoplasmic and does not alter p53 and hMDM2 localisation.

Localisation of hMDMX, hMDM2 and p53 in various VH10 (A, B) and HER (C, D) cell lines was determined by immunofluorescence using the indicated antibodies. DAPI staining was used to visualise nuclei, GFP signal represents SV-40 small-t expression.
Additional File 2. Karyotyping of VH10 and HER cell lines.
Karyotypes of transformed VH10 (A) and HER (B) cell lines using combined binary ratio labeling-fluorescence in situ hybridization (COBRA-FISH). Representative karyograms after COBRA-FISH hybridization are shown for each cell line.
Additional File 3. hMDMX overexpression inhibits p53 response but does not rescue the growth inhibition induced by 5-fluoro-uracil or etoposide in human fibroblasts.
A. The indicated VH10 and HER cell lines were treated with 10 μM Nutlin-3 for 24 hours and analyzed with qRT-PCR. Expression levels of E2F1, CDC25a (upper panel) and p73 (lower panel) were normalized for housekeeping genes RPS11 and CAPNS1.
B. Indicated VH10 cell lines were treated for 24 hours with 25 μM 5-FU, 5 μM etoposide or mock treated, and analyzed with qRT-PCR. Expression levels of hMDM2-p2 and p21 were normalized for housekeeping genes CAPNS1 and SRPR.
C. Protein levels of cells treated as in B were analyzed with immunoblotting using the indicated antibodies. D. Cell growth was monitored using WST-1 proliferation assays. 24 hours after seeding the cells were treated for 24 hours with the indicated drugs. Cell proliferation was measured at day 0, 2 and 4 after treatment.
Chapter 5

Alternative splicing of HDMX predicts p53 inactivation and bad prognosis

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Adapted version submitted for publication in PNAS
Chapter 5

Abstract

Tumour suppressor p53 is functionally attenuated in virtually all human tumours. In osteosarcoma, only ~20% of tumours contain a p53 mutation and 10-35% has amplification of p53-inhibitor HDM2 (MDM2), suggesting alternative p53-inhibiting mechanisms in the remaining tumours, such as overexpression of another p53 inhibitor, HDMX (MDMX/MDM4). An oncogenic function of HDMX in other tumour types has been shown. Expression of an alternative HDMX splice variant (HDMX-S) is correlated with worse survival and high tumour grade in several tumour types. In this study we analyzed HDMX expression and alternative splicing in high-grade osteosarcomas and osteosarcoma cell lines. We found increased HDMX-S expression in a high number of osteosarcoma, correlating with shorter disease-specific survival and increased occurrence of metastases. Analysis of osteosarcoma cell lines showed a clear association of high HDMX-S expression with mutant or inactivated p53 and low HDMX protein expression. These findings were confirmed and validated in two independent panels of tumour cell lines, the NCI60 and a set of breast cancer cell lines. The clinical meaning of these findings was emphasized by the better prognostic value of the HDMX-S / HDMX-FL ratio than p53 mutational status in a cohort of soft tissue sarcoma patients.

Using RNAi techniques to reduce HDMX levels in osteosarcoma cell lines, we observed a largely p53-independent growth inhibition and p53-dependent increased Nutlin-3-sensitivity. Furthermore, relatively high levels of full-length HDMX protein may be associated with cisplatin and doxorubicin resistance of osteosarcoma cells. Our results indicate a p53-independent role for HDMX in osteosarcomagenesis, whereas in later stage and clinically aggressive tumours a switch occurs towards the splice-variant HDMX-S, thereby reducing HDMX-FL protein levels. This altered ratio may serve as a new predictive biomarker for p53 inhibition in diverse tumour types.
**Introduction**

Osteosarcoma is the most common primary bone malignancy in children and adolescents, occurring mostly in patients between 10 and 25 years and found in the rapid growth areas of the long bones [Raymond et al., 2002]. The vast majority of osteosarcoma arises without clear hereditary setting, while a small subset occurs in the context of Li-Fraumeni syndrome or progeria syndromes. Overall survival for patients with non-metastatic osteosarcomas has been increased from 10 to 65% over the past 20 years, due to improved surgical techniques and multidrug chemotherapy, but further improvement is lacking [Lewis et al., 2007]. However, 25-50% of the patients will have a relapse or will present metastasis and survival for metastatic osteosarcomas is only 10-20% [Buddingh et al., 2010; Gelderblom et al., 2011]. Alternative therapeutic targets are required to complement the existing treatments to increase the survival of osteosarcoma patients.

In approximately 50% of all human cancers mutations are found in the TP53 gene, encoding the tumour suppressor protein p53 [Hollstein et al., 1991; Hainaut and Hollstein, 2000; Goh et al., 2011] and its activity is attenuated in many wild-type p53 tumours [Vogelstein et al., 2000]. Only 20% of high-grade osteosarcoma have p53 mutations [Yokoyama et al., 1998; Park et al., 2004; Wunder et al., 2005], while DNA of the negative regulator of p53, HDM2, an E3 ubiquitin ligase for p53 [Kawai et al., 2003; Meulmeester et al., 2003], was found to be amplified in 10-35% of osteosarcoma and mutations in p53 and HDM2 amplification were mutually exclusive [Yokoyama et al., 1998; Park et al., 2004; Wunder et al., 2005; Ito et al., 2011]. In many studies on p53 as a prognostic marker for osteosarcoma, HDM2 analysis was not included, thereby possibly underestimating the influence of p53 inactivation on prognosis. Even more, p53 activity might also be inhibited by HDMX, the near homolog of HDM2, which also functions as an essential inhibitor of p53 [Shvarts et al., 1996; Marine and Joehmsen, 2005]. Not much is known about the HDMX status in osteosarcoma, whereas for several other tumour types it has been reported that HDMX does exert an oncogenic function. Overexpression and amplification of HDMX has been shown both in tumour samples [Riemenschneider et al., 2003; Danovi et al., 2004] and tumour cell lines, mostly correlating with wild-type p53 status [Ramos et al., 2001]. Strikingly, retinoblastoma and Ewing sarcoma, tumour types that mostly retain wild-type p53, show very frequently increased HDMX expression [Laurie et al., 2006; Pishas et al., 2010].

Recently, HDMX amplification was reported in soft tissue sarcomas (16%) and osteosarcomas (35%) [Ito et al., 2011]. Earlier, Bartel et al. already found an association in
soft tissue sarcomas between \textit{HDMX} gene amplification (in 17\% of soft tissue sarcomas) and poor prognosis [Bartel et al., 2005]. Furthermore, mRNA expression of an alternative splice variant of \textit{HDMX}, \textit{HDMX-S}, was found to be upregulated in 14\% of STS samples and the increased \textit{HDMX-S}/\textit{HDMX-FL} ratio correlated with tumour grade and decreased patient survival, independent of \textit{HDMX-FL} levels. This ratio was also found to be increased in high-grade glioblastomas [Riemenschneider et al., 2003] and papillary thyroid carcinomas [Prodosmo et al., 2008]. The \textit{HDMX-S} transcript contains an internal deletion of 68 nucleotides caused by skipping of exon 6 [Rallapalli et al., 2003], resulting in 26 unique C-terminal amino acids and a premature stop codon (Fig.1A). The HDMX-S protein consists mainly of the p53 binding domain and was shown in overexpression studies to bind and inhibit p53 more efficiently than HDMX-FL. \textit{HDMX-S} expression was found increased in transformed- and serum-stimulated normal cells [Rallapalli et al., 1999]. Although these studies suggest an oncogenic role for HDMX-S, hardly any data are available on the level and function of the endogenous HDMX-S protein.

In this study we analyzed clinical osteosarcoma samples and osteosarcoma cell lines for \textit{HDMX} mRNA expression and splicing, and manipulated HDMX protein levels in a set of osteosarcoma cell lines to investigate a putative oncogenic role of HDMX in osteosarcoma. We found an association between elevated \textit{HDMX-S} mRNA levels and inactivated or mutant p53, which predicted a worse survival and enhanced risk for metastasis formation in osteosarcoma. Importantly, the correlation between the p53 functionality and alternative \textit{HDMX} splicing was confirmed in two independent sets of tumour cell lines, the NCI60 panel and a set of 39 breast cancer cell lines. Analysis of a set of soft tissue sarcoma revealed that the relatively enhanced \textit{HDMX-S} expression is a better prognostic marker than p53 mutational status, indicating the clinical relevance of the \textit{HDMX}-splicing.
Materials and Methods

Patient Material
Clinicopathological data of a group of 51 high-grade osteosarcoma patients are shown in Table 1. The majority of the tumors were of the osteoblastic histological subtype and 82% of the patients was between 10 and 25 years old at diagnosis. Part of the samples was previously described [Buddingh et al., 2011]. Biopsies were taken before pre-operative chemotherapeutics were administered. Difference in response to chemotherapy was classified as good or poor response, using the Huvos criteria [Huvos, 1991]. All tissue samples were handled according to the National Ethical Guidelines (‘Code for Proper Secondary Use of Human Tissue in The Netherlands’, Dutch Federation of Medical Scientific Societies). Soft tissue sarcoma patient material was described previously [Bartel et al., 2005].

Cell Culture and Reagents
Cell lines were maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics. Most of the osteosarcoma cell lines have been described previously [Ottaviano et al., 2010] and recently characterized with regard to phenotype and metastatic behaviour in vivo [Mohseny et al., 2011]. Osteosarcoma cell lines L2531, L2635, L2792, L2826, L2857 and L2962 were recently established at the Dept. of Molecular Cell Biology, LUMC in the lab of Dr. Szuhai. Normal osteoblasts were obtained as described previously [Cleton-Jansen et al., 2009]. Cells were treated with Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA), doxorubicin (Sigma-Aldrich, St Louis, MO, USA ) or cisplatin (Sigma-Aldrich, St Louis, MO, USA) for the indicated times and concentrations.

Plasmids, Lentiviral Transduction
The construction of lentiviral vectors expressing specific shRNAs and the production of lentivirus particles have been described recently [Lam et al., 2010]. Information and sequences of shRNAs are available in Supplementary Information.

Proliferation assay, Colony Forming assay, FACS analysis
For proliferation assays 2000-2500 cells were seeded in a 96-wells plate, and the assays were performed as described earlier [Lam et al., 2010]. For colony forming assays 3000-4000 cells were seeded in duplicate per condition in 6-wells plates; cells were treated for three days with 3 µM Nutlin-3 or mock treated and fixated and stained eight days after seeding. Colonies were quantified with the Odyssey Infrared Imager and Odyssey 2.1 analysis software (LI-COR Biosciences, Lincoln, Nebraska USA).
For FACS analysis 100,000 cells were seeded in 6-cm dishes 24 hours before mock or Nutlin-3 treatment. Cells and medium were harvested after 48 hours of treatment, fixated and analyzed on the Flow cytometer (Beckton Dickinson, Franklin Lakes, NJ USA) using
Chapter 5

FACS DIVA software after incubation in PBS with 50 µg/ml propidium-iodide (PI) and 50 µg/ml RNase.

RNA isolation, Reverse Transcription, Semi-Quantitative PCR and Q-RT-PCR
RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer’s protocol, followed by cDNA synthesis following standard protocols. Semi-Quantitative PCR was performed according to standard protocols; primer sequences are available in Supplemental Information. Band intensities were quantified using Odyssey 2.1 analysis software (LI-COR Biosciences, Lincoln, Nebraska USA). Quantitative-Real-Time PCR (q-RT-PCR) amplification of cDNA derived from frozen osteosarcoma material and manipulated cell lines was performed on the ABI 7900HT thermocycler, using FastStart Universal SYBR-Green Master (ROX) mix (Roche Biochemicals, Indianapolis, IN, USA). Details and primer sequences are available in Supplemental Information.

The cDNAs for NCI60 cell lines panel were obtained from National Cancer Institute (NCI)/NIH Developmental Therapeutics Program. qRT-PCR amplification of all cell line cDNA was performed in triplicate duplex reactions with Applied Biosystem 7500 detector according to manufactures recommendations using FAM labelled TaqMan probes (Applied Biosystems), HDMX-FL (Hs00967241_m1; exon 5-6) and HDMX-S (custom made HDMX-S_F: 5’-GCCCTCTCTATGATATGTAAGAAATC-3’; HDMX-S_R: 5’-TTCTGTAGTTCTTTTTCTGGAAAGAATC-3’; HDMX-S_M FAM: 5’-CTGCACCTTTGCTGTAGTAGC-3’). Relative gene expression was normalized according to expression of GAPDH measured in same reaction with VIC labelled TaqMan probe (4326317E, Applied Biosystem).

Measurements of HDM2 transcript levels were performed by qRT-PCR using commercially available RoboGene®MDM2 cDNA quantification module, with ABI PRISM 7000 Sequence Detection System. HDM2 measurements were normalised to GAPDH transcript levels.

Protein extraction, Western blotting and Antibodies
Protein extraction and immunoblotting were performed as described previously [Lam et al., 2010]. The antibodies are described in Supplementary Information.

Statistical Analysis
Data are presented as the mean±S.D. of duplicates or triplicates, and represent at least two independent experiments. Differences between various groups were evaluated either by one-way ANOVA followed by Bonferroni's Multiple Comparison Test or two-tailed unpaired Student’s t-Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Survival analysis was done using the Kaplan-Meier and the Cox's multivariate proportional hazards regression model with GraphPad software. Patients who were alive at the time of the last follow-up were included as censored data into the survival analyses.
HDMX splicing predicts p53 inactivation and bad prognosis

Table 1. Clinicopathological data of 51 high grade osteosarcoma patients

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<th>ToM</th>
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<td>11</td>
<td>LUMC</td>
</tr>
</tbody>
</table>

Time: all shown in months; ToM: time of detection metastasis, measured from diagnosis; ToD: time of death, measured from diagnosis; ToF: time of follow up, measured from diagnosis; WWU: Westfälische Wilhelmsuniversität, Münster, Germany; IOR: Istituti Ortopedici Rizzoli, Bologna, Italy; LUMC: Leiden University Medical Centre, The Netherlands.
Chapter 5

Results

**HDMX-S mRNA is relatively high expressed in osteosarcoma**

Clinical data of 51 fresh frozen osteosarcoma biopsies are shown in Table 1. Evaluation of HDMX mRNA expression of osteosarcoma samples, normal osteoblasts and MCF-7 cells revealed predominant HDMX-S expression in 75% (38/51) of osteosarcoma samples, whereas in normal osteoblasts and MCF-7 cells mainly HDMX-FL is expressed (Fig. 1B and Table 2).

### Table 2. Ratio between HDMX-S and HDMX-FL mRNA in 51 osteosarcomas samples.

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</tr>
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<tr>
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</table>

PCR bands representing HDMX-FL and HDMX-S shown in Fig.1B were quantified with densitometry and the ratio HDMX-S / HDMX-FL for each sample was calculated.

* Upper band below detection limit

To find out if this ratio showed any correlation with sensitivity to pre-operative chemotherapy, the ratio was compared between good (Huvos grades 3 and 4, ≥90% necrosis after chemotherapy) and bad responders, but no significant difference was found (Supp. Fig. 1A). However, in the group of patients that express mainly HDMX-FL, there is a significantly longer disease specific survival compared to the HDMX-S group (104 vs. 58 months, P= 0.0153, Unpaired t test, for patients died within or followed at least for 3 years) (Table 3).
Table 3. Analysis of clinicopathological data and $HDMX$ mRNA expression.

<table>
<thead>
<tr>
<th>Ratio $HDMX$-FL / $HDMX$-S</th>
<th>$HDMX$-FL &gt; $HDMX$-S</th>
<th>$HDMX$-S ≤ $HDMX$-FL</th>
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<tr>
<td>Total</td>
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<td>38</td>
</tr>
<tr>
<td>Male/Female</td>
<td>5/8</td>
<td>23/15</td>
</tr>
<tr>
<td>Response</td>
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<td></td>
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<td>Huvos 1</td>
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<tr>
<td>Huvos 2</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>total bad response</td>
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<td>26 (68%)</td>
</tr>
<tr>
<td>Huvos 3</td>
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<td>7</td>
</tr>
<tr>
<td>Huvos 4</td>
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<td>4</td>
</tr>
<tr>
<td>total good response</td>
<td>5 (38%)</td>
<td>11 (29%)</td>
</tr>
<tr>
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<td>1 (7.7%)</td>
<td>1 (2.6%)</td>
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<tr>
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<td>15</td>
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<td>5 year cut-off</td>
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<td>9</td>
<td>14</td>
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</table>

Calculated mRNA ratios between $HDMX$-S and $HDMX$-FL were compared between different groups within the group of 51 osteosarcoma samples.

Results of Kaplan-Meier analysis for overall survival indicated a trend for high $HDMX$-S levels associating with worse survival (Supp. Fig. 1B). Furthermore, Cox multivariate regression survival analysis (Supp. Fig. 1C), that was adjusted for tumour stage, showed a significant difference between the high and low $HDMX$-S group (P = 0.036). Interestingly, the group of patients that developed metastases within a follow-up time of 3 years, show a significantly higher $HDMX$-S/$HDMX$-FL ratio, (1.6 fold; P = 0.013, Unpaired t test) (Table 3 and Supp. Fig. 1D). Confirming, q-RT-PCR analysis revealed no significant increase in $HDMX$-FL levels (P= 0.99, Unpaired t test) in patients with metastasizing tumours, whereas total $HDMX$ mRNA is significantly higher (P= 0.0199, Unpaired t test). Therefore, the increase in total $HDMX$ mRNA must be caused by increased $HDMX$-S levels (Supp. Fig. 1D). These observations were confirmed in the Kaplan-Meier analysis for metastasis free survival, which shows clear significant differences between the high, medium and low $HDMX$-S groups (p=0.002; Fig. 1C). Cox multivariate regression survival analysis (Fig. 1D) supported these differences (P = 0.002), indicating $HDMX$-S as an independent prognostic factor for metastasis-free survival in these tumours.

No significant differences in mRNA levels of $p53$, $HDM2$, $HDMX$-FL, total $HDMX$ and $p21$ between bad or good responders were found (data not shown). Only 6% of the samples showed increased mRNA $HDM2$ and $HDMX$ expression, suggesting a low number of $HDM2$ and $HDMX$ amplification (data not shown).
**Figure 1. Analysis of HDMX-S mRNA expression in osteosarcoma biopsies**

**Fig. 1A** Schematic drawing of HDMX protein structure (upper), containing the p53 Binding Domain (p53-BD), Acidic Domain (AD), Zinc-finger (Zn) and RING Domain. Structure of HDMX-FL and HDMX-S mRNA is depicted below. Arrows indicate the start and stop codon in both mRNA structures. Exon 6 is spliced out in HDMX-S resulting in 26 novel amino acids and a premature stop codon. **Fig. 1B** Expression pattern of HDMX-FL and HDMX-S mRNA in 51 osteosarcoma biopsies, normal osteoblasts and breast cancer cell line MCF7. Results of semi-quantitative PCR with HDMX primers in exon 3 (FW) and exon 8 (Rev), resulting in bands for both HDMX-FL and -S. **Fig. 1C** Kaplan-Meier analysis of metastasis free survival of osteosarcoma patients (n=51) using high, medium and low HDMX-S/HDMX-FL ratio as prognostic factors. Patients that were alive, but not followed for the complete time-of-follow up are indicated as censored (P = 0.002, Mantel-Cox). Patients that already developed metastases before the beginning of observation time were excluded from the analysis (n=6). **Fig. 1D** Results analyzed in Fig. 1C were confirmed by Cox multivariate regression survival analysis, controlled for tumour staging (P= 0.004).
Analysis of osteosarcoma cell lines

A number of established and freshly cultured osteosarcoma cell lines, of which a subset was recently characterized regarding p53 status/expression, *HDM2* gene amplification and protein expression [Ottaviano et al., 2010], were analyzed for p53, HDM2 and HDMX protein/mRNA expression. Results are summarized in Table 4.

### Table 4. Summary of p53 status, HDM2 and HDMX levels in 22 osteosarcoma cell lines.

<table>
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<th>#</th>
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<th>HDMX</th>
<th>Rel. Quant. HDMX</th>
<th>Protein Levels</th>
<th>mRNA Levels</th>
<th>Nutlin-3 response</th>
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<td>HDMX-S</td>
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<td></td>
<td></td>
<td></td>
<td>S/FL</td>
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<td>n.a.</td>
</tr>
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<td>FL/S</td>
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<td>0.57</td>
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<td>wt</td>
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<td>FL/S</td>
<td>1.16</td>
<td>0.64</td>
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<td>FL</td>
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P53 status

P53 status was analyzed by immunoblotting of protein extracts of untreated cells and cells treated with Nutlin-3, a small molecule inhibitor of the p53-HDM2 interaction [Vassilev et al., 2004], which activates p53. Out of 22 cell lines, only 6 lines had detectable p53 protein levels and showed a functional p53 response. A huge overexpression of p53 was observed in 4 out of 22 cell lines, whereas no p53 response was observed upon Nutlin-3 treatment, suggesting the expression of a mutant p53. No or very weak p53 protein expression was found in 12 out of 22 cell lines, of which some were previously reported to contain a wild-
Chapter 5

type p53 gene (IOR/OS9, ZK-58 and MG-63) [Ottaviano et al., 2010] (Table 4 and Supp. Fig. 2A). The p53 response of p53 wild-type cell line KPD is shown in Figure 4.

HDM2 and HDMX status
Three of the six wild-type p53 cell lines, MHM, OSA and L2792, express very high HDM2 protein levels, caused by HDM2 gene amplification ([Ottaviano et al., 2010] and Szuhai et al., personal communication, 2011) and two express high levels of HDMX (U2OS and KPD; Fig. 2A). Proliferation assays and FACS experiments (Supp. Fig. 2B and C) clearly showed that high HDM2 expression correlates with a strong, apoptotic Nutlin-3 response, as reported previously [Xia et al., 2008]. HDMX overexpressing cells were relatively resistant to Nutlin-3 treatment, but stopped proliferating.

Ratio $HDMX-S / HDMX-FL$
Out of 22 analyzed osteosarcoma cell lines, 17 (77%) have a ratio $HDMX-S/HDMX-FL \geq 1.3$ (Fig. 2B and Table 4). Strikingly, in p53 wild-type cells this ratio is significantly lower (P=0.0146, Unpaired t test). These results were confirmed using quantitative RT-PCR using probes designed to detect the specific transcripts. Specifically, as the $HDMX-S$ splice variant excludes exon 6 (Fig. 1A), we used a probe that covers the exon 5/6 boundary to detect $HDMX-FL$ and a probe that covers the exon 5/7 boundary to detect $HDMX-S$. Based on HDMX and HDM2 protein expression levels, 3 groups of cell lines were made, shown in Table 4. Group 1 (High HDMX, low HDM2) and group 2 (Low HDMX, low HDM2) differ significantly in average $HDMX-FL$ mRNA expression (1.5-fold higher in group 1, P=0.034, Unpaired t test), as well as the ratio between $HDMX-S$ and $HDMX-FL$ (P=0.0034, Unpaired t test), correlating with HDMX protein levels. In group 3 (Low HDMX, High HDM2), the high HDM2 protein expression most likely is responsible for the low HDMX protein levels [de Graaf P. et al., 2003]; although $HDMX-FL$ is still moderately expressed, the level of HDMX protein is very low.

Functional p53 inhibition associates with elevated $HDMX-S$ levels in a diverse cell line panel.
The results from the analysis of the $HDMX$ transcripts in the osteosarcoma cell panel suggest that p53 inactivation is associated with an increase in $HDMX-S$ transcript and an increase in the $HDMX-S/HDMX-FL$ ratio. In order to further validate these observations in a second, independent cell panel and possibly extend these results to cancer cell lines derived from cancers other than osteosarcoma, we measured levels of the $HDMX-FL$ and $HDMX-S$ transcripts in the NCI60 cell line panel which consist of 59 cell lines from 9 distinct tumour types (Supplemental Table 1): non-small cell lung cancer (9), breast cancer...
HDMX splicing predicts p53 inactivation and bad prognosis

(5), central nervous system cancers (6), colon cancer (7), leukaemia (6), melanoma (9), ovarian cancer (7), prostate cancer (2) and renal cancer (8) [Shoemaker, 2006]. Of the 59 cell lines, 14 are known to have wild type p53, while 36 cell lines have either mutated or deleted p53 and for 9 cell lines the p53 status is inconclusive [Caron de Fromentel and Soussi, 1992; Berglind et al., 2008]. To measure the HDMX-FL and HDMX-S transcripts, we used quantitative RT-PCR using the probes described above. Interestingly and similar to the associations in the osteosarcoma cell panel, HDMX-S levels in mutant p53 are on average 2 fold higher than in cells with wild type p53 (Table 5). Specifically, cells with wild type p53 had on average delta CT measurements of 11.38 for the HDMX-S variant compared to average delta CT measurements of 10.43 in cells with mutant p53 (p= 0.0398, Unpaired t test). No significant differences in HDMX-FL were noted (p= 0.21, Unpaired t test). Therefore, as expected and predicted by the osteosarcoma cell panel data, the HDMX-S/HDMX-FL ratio is also significantly lower in cells with wild type p53 compared to the average ratio in cells with mutant p53 (p= 0.0063, Mann-Whitney test).

As mentioned above, another mechanism that cancers like osteosarcoma use to inhibit p53, other than somatic p53 mutation, is the overexpression of HDM2. We, therefore, were interested to explore if p53 inhibition by HDM2 overexpression, without p53 mutation, would also associate with elevated HDMX-S levels. To do this, we measured HDM2 transcript levels in cell lines that have retained wild type p53. Strikingly, we found the levels of the HDMX-S transcript positively correlated with HDM2 levels (correlation coefficient 0.763, Spearman, p= 0.001). Indeed, this correlation results in a 2-fold increase in HDMX-S expression those 5 cells with wild type p53 and the highest levels of HDM2 compared to those 5 cells with wild type p53 and the lowest HDM2 levels (p= 0.019, Unpaired t test, Table 5). No significant differences in HDMX-FL were noted (p= 0.907, Unpaired t test). Therefore, as expected, the HDMX-S/HDMX-FL ratio is also significantly higher in cells with wild type p53 and the highest HDM2 levels compared to the average ratio in cells with wild type p53 and the lowest HDM2 levels (p= 0.0487, Unpaired t test).

These results could be confirmed by quantification of the specific HDMX bands resulting from semi-quantitative PCR amplification (Suppl. Fig. 3A and Suppl. Table 2). Overall, when we compared the HDMX-S/HDMX-FL ratio of cells with functional active p53 (wild-type p53 and low HDM2) with that of cells that have either mutant p53 or high HDM2 levels, we found a 5-fold increase in the cells with functionally inactivated p53 (P = 0.07, Unpaired t test) and observed a significant increase of HDMX-S levels (P =0.002, Unpaired t test).
Chapter 5

Figure 2. Analysis of 22 osteosarcoma cell lines
Protein and RNA was extracted from 22 asynchronously growing osteosarcoma cell lines. Fig. 2A Protein levels were analyzed with immunoblotting using the indicated antibodies. USP7 expression was analyzed as loading control. Fig. 2B 22 osteosarcoma cell lines and 1 osteoblast sample were examined for HDMX-FL and HDMX-S mRNA expression using primers for HDMX exon 3 (Fw) to exon 8 (Rev). GAPDH mRNA expression was examined as an internal control.

Table 5. Functional p53 inactivation is associated with elevated HDMX-S levels in the NCI60

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<th>HDMX-FL*</th>
<th>HDMX-S*</th>
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Q-RT-PCR analysis was performed on cDNAs of the NCI60 cell line panel, using specific probes for HDMX-FL and HDMX-S, as described in Material and Methods section. Delta CT values were calculated using GAPDH as a housekeeping gene. Delta CT and the reciprocal ratios delta CT HDMX-S/HDMX-FL are displayed for p53 wild-type or mutant cell lines and for high or low HDM2 expressing p53 wild-type cells. P-values of differences between groups were calculated using unpaired T-Test or Mann-Whitney test, as indicated. * average delta CT; ** Mann-Whitney test; *** Unpaired t test

p53 inactivation associates with elevated HDMX-S levels in a breast cancer cell line panel.
To further validate these observations, we analysed the HDMX-FL and HDMX-S mRNA expression in a set of 39 breast cancer cell lines, previously described [Hollestelle et al., 2009] (Supp. Fig. 3B and Supp. Table 3). Again we found a significantly higher HDMX-
HDMX splicing predicts p53 inactivation and bad prognosis

The mRNA ratio $HDMX-S/HDMX-FL$ is a better prognostic factor than p53 mutational status.

To find out more about the clinical meaning of these results, we re-analysed the data of a set of soft tissue sarcomas, which were previously analysed for HDMX-S expression and p53 status [Bartel et al., 2005]. Preliminary data indicate that the ratio $HDMX-S/HDMX-FL$ is a better prognostic factor for overall survival than p53 mutation status. Kaplan-Meier analysis for overall survival using p53 mutation as a prognostic factor yields only small differences ($P = 0.509$, Kaplan-Meier) (Supp. Fig. 3C), whereas using high, medium or low $HDMX-S/HDMX-FL$ ratio clearly separates the three groups ($P= 0.013$, Kaplan-Meier). Cox multivariate regression survival analysis confirms these results ($P= 0.005$, Cox) (Supp. Fig. 3D).

HDMX inhibits basal expression of p53 targets in the osteosarcoma cell line KPD.

To elucidate a possible role of HDMX in osteosarcoma cells retaining wild-type p53, we performed HDMX-knockdown experiments in the osteosarcoma cell line KPD, which has a relatively high HDMX protein expression. To be able to assess a function for p53-regulation upon HDMX knockdown, initially a stable p53-knock-down KPD-derivative was generated, with the appropriate control. Subsequently, these cells were transduced with either HDMX- or a non-targeting control knock down virus, and the effects of decreased HDMX levels on cell proliferation and response to stress were assessed. Knockdown of HDMX was performed with two short hairpin RNA constructs, sh-HDMX#1 and sh-HDMX-3’-UTR. Either alone or combined the HDMX knockdown constructs resulted in a relative good knockdown (Fig. 3A). Confirming the role of HDMX as an inhibitor of p53, decreasing HDMX resulted in increased mRNA levels of $HDM2-P2$, $p21$ and $GADD45$-alpha, all transcriptional targets of p53 (data not shown). Accordingly, protein levels of HDM2, p21 and p53 are slightly induced, whereas in p53 knockdown cell-lines these effects are strongly diminished (Fig. 3A).

HDMX knockdown inhibits growth of osteosarcoma cell lines independent of p53.

To assess the effects of HDMX knockdown on cell proliferation and survival, both short- and long-term growth assays were performed. In a short-term growth assay, HDMX-knockdown significantly inhibited cell growth ($P<0.0001$), resulting in a relative survival
between 50 and 60% three days after seeding (Fig. 3B). Surprisingly, the observed growth inhibition appeared to be independent of p53 (P<0.0001 for p53-knockdown cells). Accordingly, this was not only observed in KPD cells, which express high levels of HDMX, but also in U2OS cells (Supp. Fig. 4A), OSA, MHM cells and in two cell lines with inactive or mutant p53, OST and HAL, respectively (Supp. Fig. 4B). In long-term growth assays (colony formation), knocking down HDMX in KPD cells results in a rather strong reduction in the number of colonies formed (P<0.0001), which appears to be partly p53-dependent (Fig. 3C).

FACS analyses showed a slight decrease of the number of cells in S-phase, which was accompanied by a small increase of cells in G2/M and sub-G1 phase upon HDMX knockdown. In cells with reduced p53 levels, this G2 arrest was even somewhat enhanced, again indicating a p53-independent function of HDMX in these cells (Fig. 3D).

Reducing HDMX levels sensitizes the osteosarcoma cell line KPD for Nutlin-3 induced growth inhibition.

HDMX inhibits p53 transcriptional activity in a similar way as HDM2, but has a much lower affinity for Nutlin-3 [Laurie et al., 2006]. Therefore, Nutlin-3 treatment might be less effective in tumours with high HDMX levels.

Control- and HDMX- knockdown KPD cells were treated with Nutlin-3, leading to a p53 response. Consequently, protein and mRNA levels of p53 targets HDM2 and p21 increased and HDMX protein levels slightly decreased after 48 hrs of treatment; all these effects are strongly reduced in p53-knockdown cells. When HDMX levels are reduced, no significant differences in changes of HDM2 and p21 at the protein and RNA level upon Nutlin-3 treatment are observed (Fig. 4A and B). However, the pro-apoptotic protein PUMA, is somewhat stronger upregulated in the cells with reduced HDMX levels (Fig. 4A). Consistently, HDMX knockdown sensitizes KPD cells for Nutlin-3-mediated growth inhibition, observed both in short-term (P<0.01) and long-term assays (P<0.01) (Fig. 4C-E). Furthermore, FACS analysis of cell cycle distribution showed that HDMX knockdown enhances the loss of S-phase cells and the number of G2/M cells in Nutlin-3 treated cells, which is diminished by concomitant p53 knockdown (Fig. 4F). Importantly, reducing HDMX levels strongly sensitizes the KPD cells for Nutlin-3 induced apoptosis as illustrated by the strong increase in sub-G1 cells, in a p53-dependent manner.

In conclusion, the relatively high HDMX levels in the KPD osteosarcoma cell line do significantly impact on the efficacy of Nutlin-3 mediated growth-inhibition, most likely by attenuation of the p53-mediated apoptotic response.
HDMX splicing predicts p53 inactivation and bad prognosis

Figure 3. HDMX inhibits basal p53 activity and stimulates the growth of the osteosarcoma cell line KPD, independent of p53

Stable control- and p53-knockdown KPD cell lines were established and lentiviral transduced with either control-, two different HDMX knockdown constructs or a combination of both knockdown vectors. Cells were counted four days post-infection and seeded for various experiments. Fig. 3A Two days after seeding, protein was extracted and analyzed with immunoblotting using the indicated antibodies. USP7 expression was analyzed as loading control. Fig. 3B Proliferation of control- and p53-knockdown cells transduced with a control- or one of two HDMX knockdown vectors was measured 72 hours after seeding and is depicted as relative survival to the corresponding control cell line. Fig. 3C Colony formation of transduced cells was measured eight days after seeding and displayed as relative to the corresponding control cells. Fig. 3D FACS analysis was performed five days post-infection on stable control- or p53-knockdown KPD cells, transduced with either control- or two distinct HDMX knockdown constructs; representative pictures for HDMX knockdown are shown. Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1. Differences between various groups were evaluated by one-way ANOVA followed by Bonferroni’s Multiple Comparison Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 4. HDMX plays a minor role in the Nutlin-3 induced p53 activation in U2OS cells

Stable control- and p53-knockdown KPD cells were lentiviral transduced with control- or a combination of two different HDMX knockdown constructs. At day 5 post-infection, cells were either mock- or Nutlin-3 (10 µM) treated for 24 and 48 hours. Protein extracts were isolated and analyzed with immunoblotting using the indicated antibodies. USP7 expression was analyzed as loading control. Representative blots are shown in Fig. 4A.

Fig. 4B mRNA expression levels of HDM2-P2 and p21 of mock- and 24 hours Nutlin-3 (10 µM) treated cells were quantified using q-RT-PCR and are shown as normalized relative mRNA levels. Fig. 4C Proliferation of cells treated for 24 hours with 10 µM Nutlin-3 was measured 72 hours after start of treatment and is depicted as relative survival to the corresponding mock-treated cells. Differences between various groups were evaluated by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Fig. 4D At day 4 post-infection, cells were seeded for colony forming assays. Cells were treated with Nutlin-3 (3 µM) for 24 hours one day after seeding and colony formation was monitored eight days later. Colonies were quantified and displayed as relative colony formation to mock-treated cells. Representative pictures of the formed colonies are shown in Fig. 4E. Fig. 4F FACS analysis was performed on KPD cells transduced with the indicated knockdown constructs, mock- or Nutlin-3 (10 µM) treated for 48 hours. Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1. Figures shown of mock-treated cells are the same as shown in figure 3D.
Full length HDMX stimulates the growth of osteosarcoma.

To investigate the role of HDMX-S in the growth of osteosarcoma cells, we performed either total HDMX- or full-length specific HDMX knockdown, using a target sequence in exon 6 of the HDMX mRNA, which is not present in HDMX-S mRNA. For this experiment we used not only KPD but also OST cells, the latter expressing predominantly HDMX-S mRNA (Fig. 2B). It is important to note that despite the high HDMX-S mRNA levels in OST cells, only full length HDMX protein could be detected (Fig. 5A). Reducing HDMX-FL in OST cells affected cell proliferation slightly, but significantly, similar to the effect of total HDMX knockdown (Fig. 5B (left panel)). Since the OST cell line contains a mutant p53 and does not respond to Nutlin-3 treatment (Table 4, Fig. 5A), no differences in growth between control cells and cells with reduced full-length or total HDMX were observed when treated with Nutlin-3 (Fig. 5B (right panel)).

The reduction of either HDMX-FL or total HDMX in KPD cells (Fig. 5C) resulted in a comparable inhibition of cell growth (Fig. 5D (left panel) and colony formation (Fig. 5E (left panel)), correlating with the knockdown efficiency, which was somewhat lower for the HDMX-FL specific knockdown. Consistently, total and HDMX-FL specific knockdown had comparable effects on basal and Nutlin-3 induced protein levels (Fig. 5C) and Nutlin-3 sensitivity in cell proliferation and colony formation assays (Fig. 5D and E (right panels)). In conclusion, our data indicate that the growth inhibition of osteosarcoma cell lines caused by HDMX knockdown is mainly through loss of HDMX-FL.

HDMX knockdown sensitizes KPD cells for cisplatin and doxorubicin.

Control and HDMX-knockdown KPD cells were treated with either cisplatin or doxorubicin and the growth/survival response was analyzed. The growth of control KPD cells is not affected by this rather low dose of cisplatin (1 µM), but reducing HDMX levels sensitized for cisplatin treatment, independent of p53 (P<0.01 for control- and P<0.05 for p53-knockdown cells) (Fig. 6A (left panel)). Accordingly, cisplatin did not significantly induce p53, p21 and HDM2 protein levels. Only a minor reduction of HDMX protein in KPD cells is observed (Fig. 6B), most likely due to enhanced degrading activity of HDM2, since HDMX mRNA levels remain constant (Fig. 6C). HDMX knockdown in KPD cells increased the cisplatin-induced mRNA levels of the pro-apoptotic p53 targets PUMA (P<0.01) and GADD45-alpha (P<0.05), whereas the induction of cell-cycle regulator p21 and of HDM2-P2 is not significantly altered (Fig. 6D).
Figure 5. Full length HDMX stimulates the growth of osteosarcoma.
OST and KPD cells were lentiviral transduced with control-, total HDMX- or full length-specific HDMX-exon 6 knockdown constructs. At day 5 post-infection, cells were treated with 10 µM Nutlin-3 for the indicated times or mock-treated and protein extracts were isolated. Fig. 5A Protein extracts of transduced OST cells were analyzed with immunoblotting using the indicated antibodies. The MX-82 anti-HDMX antibody is able to detect both full length HDMX and HDMX-S. USP7 expression was analyzed as loading control. * indicates an a-specific band, FL indicates size of full length HDMX, S indicates the region HDMX-S protein was expected to be detected. Transduced OST cells were seeded for a proliferation assay and mock- or Nutlin-3 treated for 24 hours. Proliferation was measured 72 hours after start treatment. Proliferation of mock treated cells (left panel) is displayed as survival relative to control infection of the corresponding cell line. The effect of Nutlin-3 on the proliferation (right panel) is displayed as survival relative to the corresponding mock-treated cells.

Fig. 5C Transduced KPD cells were treated with 10 µM Nutlin-3 or mock treated one day after seeding and protein extracts were isolated 24 hours later. Protein extracts were analyzed with immunoblotting using the indicated antibodies; USP7 expression was analyzed as loading control. Fig. 5D Transduced KPD cells were seeded for a proliferation assay, mock- or Nutlin-3 (10 µM) treated for 24 hours, and proliferation was measured 72 hours after start treatment. Proliferation of mock-treated cells (left) is displayed as survival relative to control
HDMX splicing predicts p53 inactivation and bad prognosis

Infection of the corresponding cell line. The effect of Nutlin-3 on the proliferation (right) is displayed as survival relative to the mock-treated cells of the same transduction. **Fig. 5E** Transduced KPD cells were seeded for colony forming assays, mock- or Nutlin-3 (3 µM) treated for 24 hours and colony formation was quantified 8 days later. Colony formation is displayed relative to control infection (left) or mock-treated cells of the same infection (right). Differences between various groups were evaluated by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Whereas cisplatin caused no growth inhibition in control cells, treatment of KPD cells with 0.5 µM doxorubicin for 24 hours resulted in dramatic, partially p53-dependent growth inhibition. Nevertheless, HDMX-knockdown cells are even more sensitive for doxorubicin (P<0.0001) (Fig. 6A (right panel)) and the enhanced sensitivity is partially p53-dependent (P<0.1 for p53-knockdown cells). This concentration of doxorubicin causes highly increased p53 protein levels, good induction of HDM2 and p21 levels on both protein and RNA level and degradation of HDMX protein (Fig. 6B and D). Interestingly, we also observed a decrease in HDMX-FL mRNA levels, accompanied with increasing HDMX-S expression (Fig. 6C). The induced protein levels of p53 and HDM2 are similar in the control and HDMX-knockdown cells, whereas p21 protein levels are slightly higher in HDMX-knockdown cells, correlating with mRNA levels (P<0.05 for p21) (Fig. 6D). Furthermore, in HDMX-knockdown cells we observe higher doxorubicin-induced levels of PUMA (P<0.01) and GADD45-alpha (P<0.05).

In conclusion, reducing HDMX protein levels in the KPD osteosarcoma cell line does slightly, but significantly enhance the sensitivity for cisplatin and doxorubicin.
Figure 6. HDMX knockdown slightly sensitizes osteosarcoma cells for cisplatin or doxorubicin treatment

Stable control and p53 knockdown KPD cells were lentiviral transduced with control- or HDMX-knockdown constructs. At day 5 post-infection, cells were treated with 1 µM cisplatin or 0.5 µM doxorubicin for 24 hours or mock treated. **Fig. 6A** Survival of cisplatin- (left) or doxorubicin- (right) treated cells was measured 72 hours after start of the treatment and depicted as relative survival compared to the corresponding mock-treated cells. **Fig. 6B** Protein extracts were analyzed with immunoblotting using the indicated antibodies; USP7 expression was analyzed as loading control. **Fig. 6C** mRNA was isolated and analyzed using semi-quantitative RT-PCR using primers in HDMX exon 3 (Fw) to exon 8 (Rev), resulting in bands for both HDMX-FL (FL) and HDMX-S (S). mRNA expression of GAPDH was investigated as an internal control. **Fig. 6D** mRNA expression levels of p21, HDM2-P2, PUMA and GADD45-alpha were quantified using q-RT-PCR and are shown as normalized relative mRNA levels. Differences between control- and HDMX-knockdown cells were evaluated by two-tailed unpaired Student’s t-Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
In this study, the putative oncogenic role of the p53 inhibitor HDMX in osteosarcoma was investigated. A role for HDMX in osteosarcoma formation was suggested by the relatively low frequency of p53 mutations and HDM2 amplification in this type of tumours [Yokoyama et al., 1998; Park et al., 2004; Wunder et al., 2005; Ito et al., 2011]. In several tumour types, amplification and overexpression of HDMX was correlated with p53 wild-type status [Riemenschneider et al., 1999; Danovi et al., 2004; Laurie et al., 2006; Pishas et al., 2010], tumour grading [Riemenschneider et al., 2003] and worse prognosis [Bartel et al., 2005]. Furthermore, a number of studies report increased mRNA expression of $HDMX-S$ in various cancers [Riemenschneider et al., 1999; Bartel et al., 2005; Prodosmo et al., 2008]. Interestingly, many tumours that showed increased $HDMX-S$ mRNA levels had down-regulated HDMX-FL, both on RNA as protein level [Bartel et al., 2005; Prodosmo et al., 2008], correlating with later tumour stage. This might indicate that HDMX expression loses its oncogenic function in highly aggressive tumours with p53 function impaired by other means than via inhibition by HDMX.

Consistent with these findings, we recently suggested that the main function of increased $HDMX-S/HDMX-FL$ ratio is to decrease HDMX-FL protein levels [Lenos and Jochemsen, 2011]. We could show that even in cells expressing high and predominantly $HDMX-S$ mRNA, only full length HDMX protein can be detected (Fig. 5A and [Lenos and Jochemsen, 2011]). Probably, the HDMX-S protein is very unstable or inefficiently translated and, therefore, most likely will not play an important, dominant role in cell proliferation. Furthermore, it was found that upon treatment with various drugs, the reduction of full length HDMX protein is accompanied by a shift from $HDMX-FL$ to $HDMX-S$ mRNA expression ([Lenos and Jochemsen, 2011] and this study).

In this study we observed predominant overexpression of $HDMX-S$ mRNA in a high number of osteosarcoma tissue samples (75%) and cell lines (77%). Especially, cells with inactive p53 and low HDMX protein levels express relatively high $HDMX-S$ mRNA. Strikingly, predominant $HDMX-S$ expression was correlated with a much worse patient survival and was significantly more frequent in clinically aggressive tumours that developed metastases within 3 years. Very recently, a subset of the cell lines mentioned in this study was analyzed for in vivo growth characteristics, including the capacity to metastasize [Mohseny et al., 2011]. One of the tested cell lines was found to produce metastases, HOS-143B, whereas the parental HOS cell line did not. Confirming our hypothesis, we find a dramatically increased $HDMX-S/HDMX-FL$ ratio and significantly decreased HDMX protein levels in HOS-143B cells compared to HOS cells (Fig. 2A and
B). Survival analysis indicated an association between high \textit{HDMX-S} expression and poor overall or metastasis-free survival. Our results in osteosarcoma were validated in independent sets of other cell lines derived from diverse tumour types, resulting again in an association between high \textit{HDMX-S} expression and mutant or inactive p53. Furthermore, we could confirm a significantly higher HDMX-FL protein expression in wild-type p53 cells, whereas no expression of the shorter HDMX-S was found.

Preliminary data in a set of soft tissue sarcoma further indicated that the ratio between \textit{HDMX-S/HDMX-FL} might serve as a better prognostic factor than p53 mutation. p53 mutational status will not include p53 inactivation by other factors, such as HDM2 amplification, whereas we show that a high ratio \textit{HDMX-S / HDMX-FL} does take such factors into account.

All in all, these data validate the significance of the observations made in the osteosarcoma cell panel and extends it to other cancers. It also supports the model that inhibition of p53 activity, by either p53 gene mutation or overexpression of \textit{HDM2}, leads to elevated \textit{HDMX-S} levels in cells. Furthermore, we have shown that a high ratio \textit{HDMX-S/HDMX-FL} may serve as a good new prognostic biomarker.

Moreover, our results are in line with previous findings in other tumours and indicate a loss of function of HDMX-FL in more aggressive, advanced stage tumours with inactivated p53. The expression of full length HDMX protein is reduced by the prevalence for the alternative splice-variant \textit{HDMX-S}, which is very inefficiently translated or produces a very unstable protein. A possible explanation for the apparent selection against HDMX expression might be that HDMX has growth inhibitory functions which are only obvious when the p53 pathway is not functional. This idea is supported by the Jones’ lab, which showed in a mouse model that in the absence of p21 or in p53-negative MEFs, MDMX has a growth suppressive role [Steinman et al., 2004; Steinman et al., 2005]. Later, it was demonstrated that MDMX suppresses transformation and tumourigenesis by promoting bipolar mitosis in p53 negative cells [Matijasevic et al., 2008].

Whereas in fresh osteosarcoma tumours only a low frequency of p53 mutation is found, establishment of osteosarcoma cell lines is apparently accompanied by strong selection for loss of p53 expression. This selection might complicate the interpretation of the analysis of these cells and is not likely to reflect the clinical situation. Only six out of the 22 cell lines (27\%) here analyzed, showed a p53-response upon Nutlin-3 treatment. Two of these express relatively high levels of HDMX (KPD, U2OS). Using these cell lines, we show in a comprehensive way that HDMX-FL overexpression may indeed play a subtle role in the inhibition of basal and activated p53 levels. When cells were treated with the p53 activating
agent Nutlin-3, HDMX expression inhibits the apoptotic response and reduction of HDMX resulted in enhanced increase of pro-apoptotic genes such as PUMA and GADD45-alpha when cells are treated with the DNA damaging drugs cisplatin and doxorubicin. Furthermore, HDMX seems to inhibit the basal levels of the p53 targets p21 and HDM2, thereby playing a role in normal cell cycle regulation.

Interestingly, we found that HDMX also fulfils a p53-independent function in stimulating the proliferation of various (p53-negative and -positive) osteosarcoma cells, illustrated by the growth inhibition caused by HDMX knockdown. Apparently alternative growth suppressing processes are inhibited by high HDMX expression. Further research to elucidate the mechanism of this novel function of HDMX in osteosarcoma is required, since it might be an interesting drug target for a subset of these tumours. Interference with this inhibitory function of HDMX will reduce cell growth and might enhance the effect of chemotherapeutic agents in osteosarcomas that are relatively resistant to p53 reactivation. Currently, several p53 reactivation therapies are in development, like adenoviral restoration of wild-type p53, mutant p53 reactivation or p53 stabilisation, some already used in clinical trials, reviewed in [Vazquez et al., 2008]. Since HDMX overexpressing tumours have been reported to be more resistant to the p53 reactivating agent Nutlin-3, the use of recently discovered HDMX inhibitors [Bernal et al., 2010] might provide new ways for osteosarcoma treatment.
Chapter 5

Acknowledgements

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Statement of Author Contributions

KLe conceived and carried out experiments and analyzed data, AG, LFG, AT and KLo carried out experiments. MLK, AMCJ, GLB and PCWH collected patient material, cell lines and clinical data and contributed to scientific discussions. AG, GLB and MLK contributed to the statistical analysis of the data. FB contributed to the collection of patient material and data analysis. KLe and AGJ designed and coordinated the study and drafted the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.
HDMX splicing predicts p53 inactivation and bad prognosis

References


Chapter 5


HDMX splicing predicts p53 inactivation and bad prognosis


## Chapter 5

### Supplemental Table 1. Summary of NCI60 cell line panel

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Summary of NCI cell line panel, including name of cell line, origin of tumour, p53 status and Q-RT-PCR data on HDM2, HDMX-FL and HDMX-S. * = inconclusive.
## HDMX splicing predicts p53 inactivation and bad prognosis

Supplemental Table 2. Semi-Quantitative PCR analysis of NCI60 panel

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Summary of NCI cell line panel, including name of cell line, p53 status and RT-PCR data on HDMX-FL and HDMX-S. HDMX-FL and HDMX-S mRNA expression was determined with RT-PCR using primers for HDMX exon 3 (Fw) to exon 8 (Rev), showed in Supp. Fig. 2A. GAPDH mRNA expression was examined as an internal control. Bands were quantified with densitometry and the ratio HDMX-S / HDMX-FL for each sample was calculated.
Chapter 5

Supplemental Table 3. Summary of breast cancer cell line panel

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</tr>
<tr>
<td>39</td>
<td>ZR75-30</td>
<td>0.47</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Summary of breast cancer cell line panel, including name of cell line, quantified levels of HDMX-FL and HDMX-S determined with RT-PCR amplification, p53 status and HDM2 and HDMX (full length) protein levels, as was published before [Hollestelle et al., 2009]. HDMX-FL and HDMX-S mRNA expression was determined with RT-PCR using primers for HDMX exon 3 (Fw) to exon 8 (Rev), showed in Supp. Fig. 3B. GAPDH mRNA expression was examined as an internal control. Bands were quantified with densitometry and the ratio HDMX-S/HDMX-FL for each sample was calculated. HDMX-FL protein bands were quantified with densitometry and USP7 expression was used as an internal control. Light-grey = p53 wild-type; dark-grey = HDM2 amplification; n.d. = not determined.
Supplemental Figures

Supplemental Figure 1. Ratio $HDMX-S / HDMX-FL$ is significantly higher in metastasizing tumours

**Supp. Fig. 1A** Ratio $HDMX-S/HDMX-FL$ as quantified from Figure 1B, plotted against good/bad response, as defined by Huvos grade. **Supp. Fig. 1B** Kaplan-Meier analysis of overall survival of osteosarcoma patients ($n=51$) using high, medium and low $HDMX-S/HDMX-FL$ ratio as prognostic factor. Patients that were alive, but not followed for the complete time-of-follow up are indicated as censored ($P = 0.231$, Mantel-Cox). Patients that already developed metastasis before the beginning of observation time were excluded from the analysis ($n=6$). Results were confirmed by Cox multivariate regression survival analysis, controlled for tumour staging ($P=0.029$)

**Supp. Fig. 1C**. **Supp. Fig. 1D** Ratio $HDMX-S / HDMX-FL$ mRNA expression was divided into a significant different non-metastatic and a metastatic group. $P = 0.0026$ (left graph). Levels of total $HDMX$ and $HDMX-FL$ specific (resp. middle and right graph) mRNA were quantified with Q-RT-PCR, $HDMX-S$ mRNA levels were calculated from this data and plotted into non-metastatic and a metastatic group. Total $HDMX$ mRNA levels were significantly different in both groups ($P=0.0152$), whereas $HDMX-FL$ mRNA levels did not significantly vary ($P=0.1337$). Differences between various groups were evaluated by one-way ANOVA followed by Bonferroni's Multiple Comparison Test.
Supplemental Figure 2. Nutlin-3 response in set of osteosarcoma cell lines

Supp. Fig. 2A Indicated osteosarcoma cell lines were tested for Nutlin-3 responsiveness by treating the cells for 0, 8 or 24 hours with 10 µM Nutlin-3, after which protein extracts were made and analyzed with immunoblotting using antibodies for HDM2, p53 and USP7. Supp. Fig 2B U2OS, KPD and OSA cells were mock- or Nutlin-3 (10 µM) treated for 24 hours, proliferation was measured 72 hours later and displayed as relative survival compared to the untreated cells. Supp. Fig. 2C FACS analysis of mock- or Nutlin-3 (10 µM, 24 hours) treated KPD, U2OS, OSA and MHM cells. Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1.
Supplemental Figure 3. Ratio HDMX-S / HDMX-FL in NCI60 panel and panel of breast cancer cell lines; high HDMX-S/HDMX-FL ratio is a better prognostic factor for overall survival in soft tissue sarcoma than p53 mutational status.

Supp. Fig. 3A Expression patterns of HDMX-FL and HDMX-S mRNA in a set of 59 cell lines from the NCI60 panel, determined by semi-quantitative PCR amplification with HDMX primers from exon 3 to exon 8 resulting in bands for both HDMX-FL and -S. GAPDH expression was used for normalization of expression levels. P53 status: wt = p53 wild-type; m = p53 mutant; i = inconclusive p53 status; wt - = p53 wild-type with low HDM2 levels; wt + = p53 wild-type with high HDM2 levels. Supp. Fig. 3B Expression patterns of HDMX-FL and HDMX-S mRNA in a set of 39 breast cancer cell lines, determined by semi-quantitative PCR amplification with HDMX primers from exon 3 to exon 8 resulting in bands for both HDMX-FL and -S. Supp. Fig. 3C Kaplan-Meier analysis of overall survival of Soft Tissue Sarcoma (STS) patients using p53 status as a prognostic factor (P = 0.509). Supp. Fig. 3D Kaplan-Meier analysis of overall survival of Soft Tissue Sarcoma (STS) patients using high, medium and low HDMX-S/HDMX-FL ratio as prognostic factor (P = 0.013). Patients that were not followed for the complete time-of-follow up are indicated as censored. Results were confirmed by Cox multivariate regression survival analysis, (P = 0.005).
Supplemental Figure 4. HDMX expression stimulates the growth of osteosarcoma cells

Supp. Fig. 4A Stable control- or p53 knockdown U2OS cells were seeded for a proliferation assay 4 days post-infection with either control- or HDMX-knockdown vectors; proliferation was measured 72 hours later. The effect of HDMX knockdown is displayed as the relative survival to the corresponding control cells. Differences between various groups were evaluated by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Supp. Fig. 4B MHM, OSA, OST and HAL cells were seeded for a proliferation assay 4 days post-infection with either control- or HDMX-knockdown vectors; proliferation was measured 72 hours later. The effect of HDMX knockdown is displayed as the relative survival to the corresponding control cells.
Supplemental Information

sh-RNA targeting sequences
shHDMX-ex1: 5'-GTTGCGGCTTCATTACT-3’
shHDMX-3’UTR: 5’-GTGCAGTGAGTCAAGATTG-3’
shHDMX-ex6#1: 5’-GGATCACAG TATGGATATT-3’
shHDMX-ex6#2: 5’-AGTCAAGCAACTGAAGC-3’

Additional plasmids used for lentiviral transduction were a non-targeting shRNA coding plasmid (SHC002, Sigma MISSION Library; Sigma-Aldrich), an HDMX 3’UTR targeting shRNA (TRCN0000003860, Sigma Mission Library) and an shRNA targeting human p53 [Brummelkamp et al., 2002] or mouse mdmx [Lam et al., 2010], which does not target the HDMX mRNA.

Semi-Quantitative RT-PCR primer sequences
For detection of both HDMX-FL and HDMX-S:
HDMX ex3 forward 5'-TGCATGCAGCAGGTGCG-3’
HDMX ex8 reverse 5’-CATTACTTCTAGGTGTAT-3’

For detection of the housekeeping gene GAPDH:
GAPDH forward 5’-AATCCCATCACCATCTTCC-3’
GAPDH reverse 5’-ATGAGTCCTTCCACGATACC-3’

Q-RT-PCR
Relative mRNA quantities were calculated using an internal standard curve of diluted pooled samples and normalized to a set of 3 control genes, CAPNS1, SRPR and TBP, with use of the SDS2.3 analysis program (Applied Biosystems).
Chapter 5

q-RT-PCR Primer sequences

HDM2-P2 Fw 5'-ACGCACGCCACTTTTTTCTCT-3'
HDM2-P2 Rv 5'-TCCGAAAGCTGGAGACCTGTGAG-3'
P53 Fw 5'-CTCTCCCCAGCCAAAGAAGAAG-3'
P53 Rv 5'-TCCAAGGCCTCAATTCAGCTCT-3'
HDMX total Fw 5'-AGGTGCAGAAGGTGAAATGTG-3'
HDMX total Rv 5'-CCATATGCTGCTTCTGCTGAT-3'
HDMX-FL Fw 5'-TCGCACAGGATCAGATGATGG-3'
HDMX-FL Rv 5'-CCCACCTCTCAAATCCAAAGG-3'
PUMA Fw 5'-GACCTCAACGCACAGTA-3'
PUMA Rv 5'-CTAATTGGGCTCCATCT-3'
p21 Fw 5'-AGCAGAGGAAGACCCATGTGGA-3'
p21 Rv 5'-AATCTGTCATGCTGTCTGCC-3'
SURVIVIN Fw 5'-GAGACAGAATAAGATGATAG-3'
SURVIVIN Rv 5'-GACAGATGGAAGGTGTGG-3'
GADD45a Fw 5'-GCGACCTGCAGTTTGCAATA-3'
GADD45a Rv 5'-ATCCCCACCTTATCCATCT-3'
CAPNS1 Fw 5'-ATGGTTTTGGAATTGACACATG-3'
CAPNS1 Rv 5'-GCTTGCCTGTGGTGTCGC-3'
TBP Fw 5'-CACGAACCACGGCAGCTGATT-3'
TBP Rv 5'-TTTTCTTGCTGCCAGTGGTGAG-3'
SRPR Fw 5'-CATGCTTTGACAGTAAACCA-3'
SRPR Rv 5'-ATTGCTTTGCATGCGGCC-3'

Antibodies used in this study:

Anti-HDMX rabbit polyclonal (A300-287A, Bethyl Laboratories, Montgomery, TX), anti-p53 monoclonal antibodies PAb1801 and DO-1, anti-HDM2 mouse monoclonal antibodies 4B2 [Chen et al., 1993] (kind gift of Dr. A. Levine) and SMP14 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21WAF1 mouse monoclonal antibody CP74 (Upstate Biotechnology), anti-HAUSP mouse monoclonal antibody 7G9 [Kessler et al., 2007] and rabbit polyclonal anti-USP7 (A300-033A, Bethyl Laboratories), anti-PUMA rabbit polyclonal antibody (Sigma-Aldrich). Secondary antibodies goat-anti-mouse-HRP and goat-anti-rabbit -HRP were obtained from Jackson Laboratories.
Chapter 6

Function and regulation of SUMOylation in the p53 pathway: a role for RNF4?

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Chapter 6

Abstract

Proper regulation of the p53 pathway is essential for normal cell proliferation, differentiation, DNA damage repair and the prevention of tumorigenesis. Post-translational modifications play an important role in p53 pathway, including modifications of p53 itself and its main regulators Hdm2 and Hdmx (also known as Mdm2 and Mdmx/Mdm4). Whereas the significance of ubiquitination in this process is evident, modification of the p53 pathway members by the small ubiquitin-related modifiers, SUMO’s, has been studied less extensively. In this study we have focused on the role of SUMOylation of p53, Hdm2 and Hdmx, the presence of a putative cross-talk between SUMOylation and ubiquitination and the role of the SUMO-specific ubiquitin ligase RNF4 in this process. We found that that under certain circumstances the SUMOylated fractions of these proteins are preferentially ubiquitinated and degraded. We also confirmed previous reports stating that relative SUMOylation of p53 decreases upon p53 activation and we found a stress-induced increased degradation of SUMOylated Hdm2, whereas Hdmx seems to be degraded in a SUMO-independent way. Although the ubiquitination of SUMOylated p53, Hdm2 or Hdmx seemed to be independent of RNF4, we showed that RNF4 overexpression stabilized transfected Hdm2 protein levels. Interestingly, RNF4 attenuated the apoptotic response upon treatment with the small-molecule p53 activator RITA. Lastly, we have shown that upon various types of treatments (e.g. IR, cisplatin) RNF4 protein levels decrease, which coincided with the apoptotic response. These results suggest the involvement of RNF4 in the apoptotic response after DNA damage and RNF4 levels in tumors might serve as a prognostic factor for the response to chemotherapeutic treatment. Moreover, this study might indicate an oncogenic function of RNF4 in certain cancers.
Introduction

The transcription factor p53 is a critical regulator of the cell fate. Upon DNA damage, p53 is rapidly stabilized and conformational changes occur to enable p53 to activate target genes leading to cell cycle arrest or apoptosis. The p53 pathway is the most commonly affected pathway in human tumors. Integrity of the p53 pathway is critical to suppress tumor formation and p53 tumor suppressor activity is decreased in virtually all tumors.

P53 stability and activity are mainly controlled via post-translational modifications including phosphorylation, acetylation and ubiquitination. Under normal growth conditions, the p53 protein is rapidly degraded via the ubiquitin-proteasome system. Ubiquitin chains are covalently conjugated to p53 by the RING finger protein Hdm2 (Mdm2), functioning as an E3 ligase. The Hdm2-homolog Hdmx (Mdmx/Mdm4) is a second RING finger protein that is critical for p53 regulation, best illustrated by the observation that mice lacking Mdmx die during embryonic development due to increased p53 activity, similar to Mdm2-deficient mice. Moreover, the significance of p53 regulation by Hdm2 and Hdmx is illustrated by the frequent occurrence of elevated levels of Hdm2 and/or Hdmx in a considerable proportion of human tumors. Despite the similarities between both proteins, Hdmx and Hdm2 exert distinct non-redundant functions in the regulation of p53. Although Hdmx has a RING domain very similar to that of Hdm2, it has not the ability to ubiquitinate p53, but mainly functions by inhibiting p53 transcription activity by binding to its N-terminal transcription activation domain.

The Hdm2 gene is a transcriptional target of p53, involved in a negative feedback-loop with p53. Upon p53 activation by DNA damaging agents, Hdm2 transcription increases leading to accumulation of Hdm2 protein. The Hdm2 protein is needed for degradation of Hdmx, which is needed for a full-blown p53-response. Conjugation of poly-ubiquitin chains to p53 by the E3 ligase Hdm2 leads to proteasomal degradation, although for poly-ubiquitination and subsequent degradation an additional function for the p300/CBP proteins has been suggested. Mono-ubiquitination is involved in altered subcellular localization or functional inhibition of p53. Several lysines on p53 have been identified as target for ubiquitination, although probably not all sites have been determined. Interestingly, many of the C-terminal lysines targeted by ubiquitination are also targets for other modifications, like acetylation and SUMOylation. Acetylation of several C-terminal lysines of p53 by p300/CBP interferes with the functional interaction with Hdm2, since deacetylation of these lysines of p53 is required for efficient degradation of p53, and conversely, ubiquitination by Hdm2 prevents acetylation of p53. In addition to the C-terminal acetylations, which...
interfere with ubiquitination, p53 is acetylated on lysines K120 and K164, which are located in the DNA binding domain, thereby inhibiting the binding of Hdm2 and Hdmx to p53 on DNA, allowing the activation of p53 transcriptional activities\(^27\). Whereas individual acetylation defects can be compensated by acetylation of the other lysines, the loss of all these acetylation sites results in a complete inhibition of p53-mediated p21 activation and growth arrest\(^27\), indicating the importance of acetylation on the regulation of the p53 response.

In addition, the conjugation of the small ubiquitin related modifiers (SUMO) can fine-tune the p53 regulation. SUMOylation of the C-terminal K386 of p53 was reported to inhibit the p53 transcriptional activity by interfering with the acetylation of that same site. In contrast, earlier reports showed an enhanced p53 activity upon SUMOylation\(^28\)\(-31\). In humans, three forms of SUMO are expressed, SUMO-1, SUMO-2 and SUMO-3, of which SUMO-2 and -3 are very similar and able to form polySUMO chains\(^32\)\(-33\). A consensus SUMO-conjugation site, \(\psi\)KxE (\(\psi\), a large hydrophobic residue; x, any amino acid) was identified\(^34\), although recent results has yielded more insight into SUMOylation that occurs at non-consensus sites\(^35\). SUMOylation has been implicated in the regulation of proteins in various ways, such as subcellular localization, activity, and degradation of target proteins\(^36\). Whereas K386 in p53\(^31\)\(-37\) and two lysines (K254 and 379) in Hdmx\(^38\) were identified to be the main SUMO target sites in these proteins, no SUMOylation sites could be defined in Hdm2, probably due to alternative conjugation sites\(^39\)\(-40\). Nevertheless, Hdm2 SUMOylation was reported to take place in the nucleus, by PIAS and RanBP2 proteins\(^41\). Deconjugation of SUMO-1 from Mdm2 by the SUMO-protease SUSP4, a mouse homologue of the human SENP2 (SSP3), has been suggested to activate the p53 response by stimulating Mdm2's autoubiquitination and degradation \(^42\). In contrast, a more recent report indicates that de-SUMOylation of Hdm2 by the SUMO protease SENP2 (SSP3), which takes place at the nuclear PML bodies, negatively regulates the p53 response after DNA damage\(^43\). In this study it is suggested that SUMO-1 conjugation to Hdm2 somehow interferes with the degradation of p53 by Hdm2. It is important to note that most of the above studies have been focusing on SUMO-1 conjugation, and hardly information is available on conjugation of SUMO-2 or SUMO-3 to e.g. Mdm2 and Mdmx. Although conjugation of SUMO-2 and SUMO-3 to p53\(\text{in vitro}\) has been shown, no functional studies have been reported to date.

Recently, a new kind of SUMO-ubiquitin interaction has been elucidated, with the discovery of ubiquitin E3 ligases that specifically target SUMOylated proteins. These STUbLs (SUMO Targeted Ubiquitin Ligases) contain SUMO Interacting Motifs, (SIMs) thereby enabling the specific targeting of SUMOylated proteins\(^44\). The Ring Finger protein
4 (RNF4) was identified as a STUbL in human, containing 4 SIM domains, thereby able to recognize specifically poly-SUMOylated proteins. The first reported target for this poly-SUMO-specific ubiquitination was the promyelocytic leukemia protein (PML), which is specifically degraded by RNF4 upon treating cells with arsenic trioxide.

SUMOylation can be regulated in various ways, for instance by regulation of the activity of SUMO E3 ligases or SUMO-specific proteases. An important role for members of the Mitogen Activated Protein Kinase (MAPK) pathway in regulation of SUMOylation has been shown. On the one hand, the phosphorylation of target proteins by activated ERK, p38 or JNK has been reported to either induce SUMOylation or prevent the SUMO conjugation. On the other hand, the MAPK pathways might also be involved in the regulation of SUMOylation by phosphorylation of SUMO E3 ligases, reviewed in Guo et al. (2007). Arsenic-induced phosphorylation of PML by the MAPK pathway, especially by the ERKs, JNKs and p38, is an important step in the RNF4-mediated degradation of PML, enhancing its SUMOylation, possibly by recruiting an E3 SUMO ligase to PML.

In this study we have investigated a putative SUMO-dependent ubiquitination of p53, Hdm2 and Hdmx and a possible role of RNF4 in this process.
Chapter 6

Material and Methods

Cell Culture and Reagents

MCF7 and U2OS cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% glutamine and antibiotics. H1299 cells were maintained in RPMI supplemented with glutamine and antibiotics. Stable MCF7 cells lines expressing His6-SUMO-2 were continuously grown under neomycin selection. For hypoxia experiments cells were maintained at 1% oxygen for at least 48 hours. Cells were treated with arsenic trioxide (Sigma-Aldrich, St Louis, MO, USA), etoposide (Sigma-Aldrich), Neocarzinostatin (Sigma-Aldrich), MG132 (Sigma-Aldrich), MEK1/2 inhibitor U0126 (Calbiochem), JNK inhibitor SP600125 (Calbiochem), cisplatin (Sigma-Aldrich), RITA (Gift from Galina Selivanova), Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA) for the indicated times and doses.

Plasmids, lentiviral transduction

Expression vectors encoding Flag-RNF4 and p-DEST-V5-RNF4 for transfection assays and the lentiviral pRRL vectors encoding Flag-RNF4-GFP, Flag-RNF4-puro, RNF4-Flag-GFP or RNF4-Flag-puro were obtained from Vertegaal and coworkers, and will be described in detail elsewhere. The V5-RNF4 Ring mutant C133S was made by site directed mutagenesis using the following primer pairs:

Forward: 5'-CCTCAGGTACTGTCAGTTCTCCCATCTGCATGGACGG-3' and Reverse: 5'-CCGTCCATGCAGATGGGAGAACTGACAGTACCTGAGG-3'.

His6-SUMO-2 and His6-Ubiquitin lentiviruses were made by inserting the His6-SUMO-2 or His6-Ubiquitin into the EcoRV site of plasmid pLV-CMV-IRES-eGFP. Other plasmids used for transfection were pcDNA3.1-HA-Hdmx and pcDNA3.1-HA-Hdmx-K254/379R, pReCMV-p53 and pReCMV-p53-K386R, pcDNA3.1-HA-Hdm2, pcDNA3.1-GFP and pcDNA3.1 empty vector.

Transfections were performed using Fugene transfection reagents, according to the manufacturer’s protocol. Production of lentiviruses by transfection into 293T cells has been described previously. Lentivirus expressing shRNA against RNF4 and control shRNA-lentiviruses (sh-luciferase, SHC007 or non-targeting, SHC002) were obtained from the Mission Library (Sigma-Aldrich). The sh-RNF4-expressing lentivirus #2 was made by cloning a short hairpin sequence containing the target sequence 5'- AACCAACAUCTGAUAUGUAAA-3' in the pRRL-puro lentiviral expression plasmid.

Cells were seeded 24 hours prior to lentiviral infection. To obtain specific knockdown, cells were infected with RNF4- or control-shRNA expressing lentiviruses. For all infections, a multiplicity of infection of ~ 1.5 (MOI 1.5) was used. Cells were transduced overnight in the presence of 8 μg/ml polybrene (Sigma-Aldrich). The next day, the medium was replaced with fresh medium. Transduced cells were seeded for experimental purposes 3 to 4 days post-transduction.

Antibodies

Antibodies used in this study were anti-Hdmx rabbit polyclonal (A300-287A, Bethyl Laboratories, Montgomery, TX), anti-Hdmx monoclonal mouse antibody MX-82 (Sigma), anti-p53 monoclonal antibodies PAb1801 and DO1, anti-Hdm2 mouse monoclonal antibodies 4B2 (kind gift of Dr. A. Levine) and SMP14 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21WAP1 mouse monoclonal antibody CP74 (Upstate Biotechnology), rabbit polyclonal anti-USP7 (A300-033A, Bethyl Laboratories), anti-RNF4 rabbit polyclonal antibody K39 (gift from Alfred Vertegaal), anti-phospho-ERK1/2 mouse monoclonal antibody (Sigma), anti-ERK1/2 rabbit polyclonal antibody (Sigma), anti-HA.11 mouse monoclonal antibody (Covance), anti-KAP1 mouse monoclonal antibody (BD Transduction Laboratories), anti-HIF1-alpha antibody (BD Transduction Laboratories), anti-vinculin mouse monoclonal antibody (Sigma-Aldrich), anti-Flag rabbit polyclonal antibody M2 (Sigma-Aldrich), anti-V5 antibody (Invitrogen), anti-GFP mouse monoclonal antibody (Roche), anti-PUMA rabbit polyclonal antibody (Sigma-Aldrich), anti-PML mouse monoclonal antibody PML-97 (Abcam).

Secondary antibodies goat-anti-mouse-HRP and goat-anti-rabbit-HRP were obtained from Jackson Laboratories.
Immunoblotting analysis

Cells were lysed in Giordano 250 buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA), supplemented with a mixture of protease- and phosphatase inhibitors. Protein content was determined using Bradford Reagent (Biorad) and equal amounts of protein were separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Membranes were blocked in Tris-buffered saline, 0.2% Tween-20 (TBST) containing 10% non-fat dry milk, and subsequently incubated with the appropriate primary antibody diluted in TBST containing 10% milk. Membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibody (Jackson Laboratories). Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce) followed by detection with the ChemiGenius XE3 system (Syngene, Cambridge, UK) or X-ray film (Fuji). Alternatively, membranes were blocked with Blocking Buffer for Fluorescent Western Blotting (Rockland), incubated with the appropriate primary antibodies, washed three times with TBST and incubated with secondary antibodies coupled to IRdye-680 and IRdye-800 near Infrared dyes (LI-COR Biosciences) and analysed with the Odyssey Infrared Imager (LI-COR Biosciences). Signals were quantified using the Odyssey 2.1 analysis software (LI-COR Biosciences).

Ubiquitination/SUMOylation assay in cells

Cell-based ubiquitination and SUMOylation assays were adapted from the established methods with the following modifications. After harvesting the cells by scraping in PBS, 20% of the cells was used for total protein extraction as described above. The remaining cells were used for His-pull-down assays, after correction for protein content. His-Ubiquitin or His-SUMO-2 pull-downs were performed with 40 µl of Ni-nitrilotriacetic acid beads (Qiagen) overnight at 4 °C in 6 ml of 6 M guanidinium-HCl, 0.1 M Na2HPO4·NaH2PO4, and 0.01 M Tris-HCl (pH 8.0) plus 10 mM imidazole and 10 mM β-mercaptoethanol (buffer A). After incubation, the beads were successively washed with 1 ml of each of the following buffers: buffer A lacking imidazole but with 0.2% Triton X-100; buffer B (8 M urea, 0.1 M Na2HPO4·NaH2PO4, and 0.01 M Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, and 0.2% Triton X-100); buffer C (8 M urea, 0.1 M Na2HPO4·NaH2PO4, and 0.01 M Tris-HCl (pH 6.3), 10 mM β-mercaptoethanol, and 0.2% Triton X-100; buffer C containing 0.1% Triton X-100. Ubiquitinated proteins were eluted in 2X Laemmli sample buffer containing 100 mM imidazole. The samples were boiled and analyzed by Western blot analysis with indicated antibodies.

RNA isolation, Reverse Transcription and q-RT-PCR

RNA was isolated from cells using the SV Total RNA Isolation System (Promega) according to the manufacturers’ protocol. cDNA was made from 1 µg of total RNA in an Reverse Transcriptase reaction containing ImPromII Reverse Transcriptase, RNasin RNase inhibitor, MgCl2 and 5x RT-Buffer (Promega). Quantification of mRNA expression was performed by Quantitative-Real-Time PCR, in an ABI 7900HT thermocycler, using the FastStart Universal SYBR Green Master (ROX) mix (Roche). Relative mRNA quantities were calculated using an internal standard curve, made from a dilution series of the pooled samples, using the SDS2.3 analysis program (Applied Biosystems). Expression levels of the mRNAs were normalized to the geometric mean of the control genes CAPNS1, TBP, SRPR. Primer sequences are available in Supplemental Information.

Proliferation assays, Drugs sensitivity assays, Colony formation assays

For proliferation assays, 2000-5000 cells were seeded in triplicate in a 96-wells plate, and the assays were performed as described earlier. Drugs sensitivity was assessed by performing proliferation assays on cells treated with a range of drug concentrations, Dose-Effect analysis was performed using the CompuSyn program. For colony forming assays 10,000 cells were seeded in duplicate per condition in 6-wells plates; cells were treated for three hrs with the indicated drug or mock-treated, after which cells were re-fed with fresh medium. Colonies were allowed to grow for eight to ten days, fixed with methanol/acetnic acid (3:1) and stained with Giemsa. Colonies were quantified with the Odyssey Infrared Imager and Odyssey 2.1 analysis software (LI-COR Biosciences, Lincoln, Nebraska USA).
Chapter 6

Results

SUMOylation of p53, Hdm2 and Hdmx.

Ubiquitin-conjugation of p53, Hdm2 and Hdmx has been implicated in the regulation of these proteins and their interactions. To study a putative role of SUMOylation in the ubiquitination of these proteins, we made use of MCF-7 cells stably expressing His6-tagged SUMO-2, enabling the purification of SUMOylated proteins using Ni-NTA beads.

In untreated His6-SUMO-2 cells, one main SUMO-p53 species is present, likely reflecting SUMOylation at p53-K386, as has been reported (Fig. 1A)\textsuperscript{31,37}. Strikingly, treatment with the proteasome inhibitor MG132, thus blocking the degradation of poly-ubiquitinated proteins, resulted in the accumulation of more, particularly high molecular weight species, most likely reflecting SUMOylated- and ubiquitinated p53 species. However, the total levels of p53 also increased strongly, suggesting that under normal growth conditions SUMOylated p53 species are not preferentially ubiquitinated and degraded. Similar results were obtained for Hdm2; an increase in high molecular weight proteins in the His-pull-down fraction upon MG132 treatment is concomitant with an increase of total Hdm2 levels. However, no SUMOylation of Hdm2 could be detected in the absence of MG132. This result could suggest that SUMOylated Hdm2 is more efficiently degraded than unmodified Hdm2. This difference in stability between SUMO-modified versus total protein is even more clear with Hdmx. Total levels of Hdmx are not affected by this relatively short MG132 treatment, since Hdmx has a long half-life in MCF-7 cells. In contrast, a strong accumulation of SUMOylated Hdmx and higher molecular weight bands is observed upon proteasome-inhibition. This result strongly indicates a higher turnover of SUMOylated Hdmx compared to the total Hdmx pool. Interestingly, total levels of KAP1 are not affected by MG132 treatment, suggesting a relatively stable protein, but SUMOylated KAP1 actually decreases. This might be the result of a lack of free SUMO-2, since most of it is now trapped in stabilized proteins (Figure 1A).

To further show the specificity of the His-pull down, we made stable MCF-7 cell lines overexpressing His6-SUMO-2 together with HA-tagged Hdmx or HA-Hdmx-K254/379R, which has both identified SUMO consensus sites mutated. Only in cells expressing wild-type HA-tagged Hdmx, SUMOylated Hdmx and a ladder of SUMOylated/ ubiquitinated species upon MG132 treatment is detected (Fig. 1B). Similarly, when we transfected murine B16 cells with His-SUMO-2 and either human wild-type p53 or K386R-p53, to enable specific detection of the human p53, we observed a major decrease of the amount of p53 pulled-down in the cells transfected with K386R-p53 in which the major SUMO-conjugation site is mutated. Some SUMOylated p53 is still detected, suggesting the
presence of a minor SUMOylation site on p53. Under these conditions, the total amount of p53 is only slightly increased upon MG132 treatment, whereas the SUMOylated fraction of p53 is increased significantly, indicating that a small fraction of p53 might be degraded in a SUMO-dependent manner (Fig. 1C (left panel)). Although K386 is an ubiquitin acceptor site as well, mutation of this site did only slightly decrease the amount of total ubiquitinated p53 (Fig.1C (right panel)), mainly correlating with the somewhat lower total mutant-p53 levels. Altogether, these results suggest that SUMOylation only plays a minor role in the regulation of the ubiquitination of p53. Nevertheless, we show here that SUMO-specific degradation of both Hdm2 and Hdmx takes place, which might indirectly play a role in the p53 regulation.

Activation of ERK1/2 plays a role in the SUMO dependent ubiquitination of p53 and Hdmx.

The MAPK signaling cascade plays important roles in the determination of the outcome of diverse stimuli. It has been shown that Hdmx mRNA expression is regulated via mitogenic stimuli, via the Raf-Ras-ERK pathway, which results in enhanced Ets-1 or Elk-1 binding to the Hdmx promoter 59. Hdm2 expression has also been shown to be influenced by the ERK-MEK pathway, by regulating the Hdm2 mRNA nuclear export 60 and, similar to Hdmx regulation, its transcription is induced by oncogenic Ras via activation of the MAPK pathway 61. Since it has been shown that activation of MAPK pathways can regulate the SUMOylation status of several proteins 49-53, we used a MEK inhibitor to prevent ERK1/2 activation and investigated the effects on the SUMOylation and ubiquitination status of the p53 pathway (Figure 2). Already 2 hours after MEK inhibition, the phosphorylation of ERK1/2 is already completely blocked (Fig. 2A). Inhibition of MEK was accompanied by a fluctuating p53 protein level and a decrease in Hdm2 total protein level. The decrease of Hdm2 levels could not be rescued by proteasome inhibition, suggesting a regulation at the mRNA level, confirming previous reports 60. We did not observe any significant changes in Hdmx levels upon MEK inhibition (Fig. 2A; left panel). Especially in the absence of MG132 the p53 SUMOylation decreases in time, suggesting a role of the MAPK pathway in the SUMOylation of p53. Furthermore, we also observed a decrease in basal SUMOylation and the levels of higher molecular weight species upon MG132 treatment of Hdm2 and Hdmx, particularly at the 24 hrs time point (Fig. 2A; right panels). Since total Hdmx levels remained constant, these results might indicate that SUMOylation of Hdmx is partially dependent of the activation of ERK1/2. Although total Hdm2 levels decrease upon ERK1/2 inhibition, the relative SUMOylation of Hdm2 seems to decrease even more, suggesting that also the SUMOylation of Hdm2 is at least partially regulated by ERK signaling.
Figure 1. p53, Hdm2 and Hdmx are subject to SUMO-dependent ubiquitination.

(A) MCF7 cells or MCF7 cells stably expressing His6-SUMO-2 were treated with MG132 (10 µM) or mock treated for 6 hours. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 purified fractions were analyzed by immunoblotting using the indicated antibodies. Tubulin expression was used as a loading control. (B) MCF7 cell lines stably expressing expression vectors encoding HA-tagged Hdmx or the SUMOylation deficient mutant HA-Hdmx-K254/379R were transfected with His6-SUMO-2. Cells were treated with MG132 (10 µM) or mock treated for 6 hours at day 1 post-transfection. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 purified fractions were analyzed by immunoblotting using an anti-HA antibody. (C) Stable murine B16 cells expressing wild-type p53 (wt) or SUMO-deficient mutant p53-K386R (KR), were lentivirally infected with an expression vector for His6-SUMO-2 or His6-Ubiquitin. At day 4 post-infection, cells were treated with MG132 (10 µM) or mock treated for 6 hours. Cells were lysed and His6-SUMO-2 or His6-Ubiquitin conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 or His6-Ubiquitin purified fractions were analyzed by immunoblotting using a mix of the anti-p53 antibodies DO-1 and PAb1801.
Preliminary results indicate that the ubiquitination of p53 and Hdmx is also decreased upon MEK inhibition, which might be the result of decreased Hdm2 levels, whereas the relative ubiquitination of Hdm2 does not change (data not shown). To find out more about the role of MAPK pathways, we compared the MEK inhibitor U0126 with the JNK inhibitor SP600125. The JNK inhibitor induced a p53 response, illustrated by the induction of p53 and Hdm2, whereas Hdmx levels decreased. These changes were reflected in the SUMOylated fractions, indicating JNK signaling is not required for SUMO-dependent ubiquitination (Fig. 2C). Summarized, we have shown here that activation of ERK1/2 might be involved in the SUMOylation of p53, Hdm2 and Hdmx; it might thereby also indirectly regulate p53 activity. However, we could not show that the regulation of Hdmx SUMOylation also affected Hdmx levels. Furthermore, the reduced SUMOylation of Hdm2 upon MEK inhibition was accompanied by a decrease in total Hdm2 levels, most likely caused by a decrease in Hdm2 mRNA level.

Role of RNF4 in the ubiquitination of SUMOylated p53, Hdm2 and Hdmx.
RNF4 was identified as a STUbL, (SUMO Targeted Ubiquitin Ligase), a class of E3 ubiquitin ligases that might specifically ubiquitinate poly-SUMOylated proteins and is essential for arsenic trioxide-induced proteasomal degradation of PML. Since we found accumulation of ubiquitinated, SUMOylated p53, Hdm2 and particularly Hdmx upon proteasome inhibition, we wondered whether RNF4 would play a role in this process. The RNF4 levels in MCF-7 cells stably expressing His6-SUMO-2 or His6-ubiquitin were reduced by transduction with RNF4-shRNA expressing lentiviruses. Cells were mock-treated or treated for 6 hours with MG132; SUMO-2 conjugated proteins were purified and analyzed by western blotting. As shown in figure 3A, the accumulation of SUMOylated (and ubiquitinated) p53 is strongly reduced in the RNF4 knock-down cells (left), whereas total p53 ubiquitination is only slightly decreased (Fig. 3B). However, levels of mono-SUMOylated and total p53 were also reduced upon RNF4 knockdown in the His6-SUMO2 cells, indicating no specific role of RNF4 in the ubiquitination of SUMOylated p53. The basal SUMOylation of Hdmx and the accumulation of high molecular SUMOylated species of Hdm2 and Hdmx upon proteasomal inhibition, all decrease after RNF4 knockdown. However, this might be just a reflection of the decreased total protein levels (Fig. 3A; right panel). Furthermore, p21 protein levels are slightly reduced upon RNF4 knockdown (Fig. 3A). These effects are likely to take place at the protein level, since no alterations in mRNA levels of P53, HDM2, HDMX and P21 were observed (data not shown). In His6-ubiquitin expressing cells, p53 levels were not significantly affected by RNF4 knock-down (Fig. 3B). In these cells, RNF4 knockdown did also not result in reduced Hdm2 levels and the reduction in Hdmx protein level was much less compared to the effect in SUMO-2
expressing cells. Altogether, these results suggest that RNF4 has no essential role in the SUMOylation and ubiquitination of SUMOylated p53, Hdm2 or Hdmx, although indirect effects on protein level cannot be excluded.

Figure 2. Activation of ERK1/2 plays a role in the SUMOylation and SUMO-dependent ubiquitination of p53 and Hdmx.

MCF7 cells stably expressing His6-SUMO-2 were treated with (25 µM) U0126 for 2, 6 or 24 hours or mock-treated; 6 hours before harvesting cells were incubated with 10 µM MG132 or mock-treated. Control MCF7 cells were treated with 25µM U0126 for 24 hours or mock-treated, 6 hours before harvesting, cells were additionally treated with 10 µM MG132 or mock-treated. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates (3A) and His6-SUMO-2 purified fractions (3B) were analyzed by immunoblotting with the indicated antibodies. Figure 3C. MCF7 cells stably expressing His6-SUMO-2 were treated with (25 µM) U0126 or (20 µM) SP600125 for 6 and 24 hours or mock-treated. Cells were treated with 10 µM MG132 or mock-treated 6 hours before harvesting. Cells were additionally treated with 10 µM MG132 or mock-treated. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates (left panel) and His6-SUMO-2 purified fractions (right panel) were analyzed by immunoblotting with the indicated antibodies.
RNF4 and SUMOylation in the p53 pathway

**Figure 3. Role of RNF4 in SUMOylation and ubiquitination of p53, Hdm2 and Hdmx.**
MCF7 cells were lentivirally infected with a His6-SUMO-2 or His6-ubiquitin expression vector and either an expression vector encoding a non-coding shRNA or a shRNA against RNF4. Four days later, cells were treated with MG132 (10 µM) or mock-treated for 8 hours. Cells were lysed and His6-SUMO-2 and His6-ubiquitin conjugates were purified using Ni-NTA beads. Total cell lysates and His-purified fractions were analyzed by immunoblotting with the indicated antibodies. (A, left panel) SUMOylation of p53 and total protein levels of p53, RNF4, p21 and USP7 of His6-SUMO-2 transduced cells. (A, right panel) SUMOylation of Hdm2 and Hdmx and total protein levels of Hdm2 and Hdmx of SUMO-2 infected cells. USP7 expression was analyzed as a loading control. (B) Ubiquitination of p53 and total protein levels of Hdm2, Hdmx, p53, RNF4, p21 and USP7 of His6-Ubiquitin infected cells.

**RNF4 overexpression stabilizes Hdm2 protein levels, RING-dependently.**
To further study a putative function of RNF4 in the p53 pathway, we overexpressed V5- or Flag-tagged RNF4 in various cell lines. We observed very slight differences in endogenous Hdm2 levels upon RNF4 overexpression, possibly because only a minor fraction of the cells were transfected. Therefore, we co-transfected Hdm2 together with V5-tagged RNF4 in p53 wild-type MCF-7 cells, p53/mdm2 double knockout MEFs, p53-negative H1299 cells and p53-/- and p53+/+ HCT116 cells. In all cases we observed an increase of exogenously expressed Hdm2 protein when RNF4 was co-transfected, which was independent of endogenous p53 expression (Fig. 4A). Similar effects were observed when HA-Hdm2 together with Flag-tagged RNF4 was used (Fig. 4B; left panel). However, level of exogenously expressed HA-Hdmx was not affected by RNF4 co-expression (Fig. 4B; right panel). Furthermore, an RNF4 RING mutant (C133S) was not able to increase Hdm2
Figure 4. RNF4 overexpression stabilizes Hdm2 protein levels in a RING-dependent manner.

(A) MCF7, p53/mdm2-/-MEFs, H1299, HCT116 p53-/- and HCT116 p53+/+ cells were transfected with a vector encoding Hdm2 (1 µg), V5-RNF4 (2 µg) or with an empty vector to a total of 3 µg per 6-cm dish, as indicated. All cells were co-transfected with 0.3 µg GFP encoding vector as a transfection control. Cells were lysed 24 hours post-transfection, total cell lysates were analyzed by immunoblotting with the indicated antibodies. (B) MCF7 and H1299 cells were transfected with 1 µg HA-Hdm2, HA-Hdmx or empty vector, together with either 1 µg (+) or 2

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(B) Figure shows immunoblot analysis of Hdm2 and RNF4 expression in MCF7 and H1299 cells after transfection.

(C) V5-RNF4 and Hdm2 expression in MCF7 and H1299 cells with or without MG132 treatment.

(D) Quantification of Hdm2 and RNF4 protein levels in MCF7 and H1299 cells.

(E) Co-immunoprecipitation of Hdm2 and RNF4 in MCF7 and H1299 cells.

(F) Western blot analysis of Hdm2, p53, V5, and USP7 in MCF7 and H1299 cells.

(G) Gel electrophoresis of proteins from MCF7 and H1299 cells.
µg (+) Flag-RNF4 encoding vector or empty vector was added to a total of 3 µg per 6-cm dish. All cells were co-transfected with 0.3 µg GFP encoding vector as a transfection control. Cells were lysed 24 hours post-transfection, total cell lysates were analyzed by immunoblotting with the indicated antibodies. (C) H1299 cells were transfected with 1 µg V5-RNF4, the RING-deficient V5-RNF4-C133S mutant or an empty expression vector, without or with co-transfection of 1 µg Hdm2. As a transfection control 0.3 µg GFP encoding vector was co-transfected. Cells were lysed 24 hours post-transfection, protein levels were analyzed by immunoblotting with the indicated antibodies. (D) RNA extracts were analyzed using q-RT-PCR and are displayed as normalized relative HDM2 mRNA levels. (E) H1299 cells were transfected with an empty expression vector or 2 µg V5-RNF4 expression vector and co-transfected with 1 µg (+) or 2 µg (++) Hdm2 encoding vector or empty vector was added to a total of 3 µg per 6-cm dish. All dishes were co-transfected with 40 ng p53 expression vector. Cells were treated with 10 µM MG132 or mock treated for 5 hours 24 hours post-transfection. Cells were lysed and protein extracts were analyzed by immunoblotting with the indicated antibodies, USP7 expression was used as a loading control. (F) MCF7 and H1299 cells were transfected with transfection vectors encoding Hdm2 (1 µg), V5-RNF4 (+ = 0.5 µg; ++ = 1 µg; +++ = 2 µg) as indicated and all were co-transfected with 1 µg His6-ubiquitin. One day post-transfection cells were lysed and His6-Ubiquitin conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 purified fractions were analyzed by immunoblotting using the indicated antibodies. (G) MCF7 cells stably expressing His6-SUMO-2 were transfected with an empty expression vector or a Flag-RNF4 overexpression vector, both 6 µg per 10-cm dish. One day post-transfection, cells were treated with MG132 (20 µM) or mock treated for 6 hours. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates and His-purified fractions were analyzed by immunoblotting with the indicated antibodies. USP7 expression was analyzed as a loading control.

levels (Fig. 4C). No significant changes in HDM2 mRNA levels occurred upon RNF4 overexpression (Fig. 4D), excluding a possible effect of RNF4 on transcription. The increase in Hdm2 protein levels is most likely caused by increased protein stability, which is dependent of an intact RING domain of RNF4. Indeed, the observed increase in Hdm2 protein levels upon RNF4 expression was absent in cells that were treated with MG132, suggesting that RNF4 overexpression somehow interferes with the proteasomal degradation of Hdm2 (Fig. 4E). Furthermore, in the same experiment, the overexpression of RNF4 seems to induce p53 protein levels, which is absent in the presence of MG132. However, no significant changes of the ubiquitinated fraction of p53 (data not shown) or Hdm2 could be detected upon RNF4 overexpression (Fig. 4F). Interestingly, in a His-pull-down of MCF7/His6-SUMO-2 cell extracts, mainly the amount of high molecular species of SUMOylated Hdm2 and p53 decreased when cells were transfected with Flag-RNF4 (Fig. 4G). However, this effect was only noticeable when cells were treated with MG132, suggesting a, most likely indirect, interfering effect of RNF4 on the ubiquitination of the SUMOylated proteins. No change in Hdmx SUMOylation was found upon RNF4 overexpression (data not shown). The basal protein levels of p53 and Hdm2 are only slightly enhanced in the RNF4 overexpressing cells, whereas MG132 treatment prevents this effect. These results are confirming that the SUMOylation of p53 only slightly affects the total p53 levels, as shown in figure 1 and 4. Furthermore, based upon these results, a direct role of RNF4 in the ubiquitination of SUMOylated p53 or Hdm2 is not likely, since then an increase of SUMO/ubiquitination would be expected upon RNF4 overexpression.
Role of SUMOylation in the p53 response upon various stresses.
Modulation of p53, Hdm2 and Hdmx levels and/or activity plays an important role in the cellular response to various types of stress, especially DNA damage. Since situations of stress will induce a p53 response, the interaction between p53 and its inhibitors needs to be attenuated. Several types of post-translation modifications, including SUMOylation, may be involved in the regulation of the functional interaction of these proteins upon stress. Therefore, the SUMOylation status of these proteins upon various stresses was studied.

SUMO-dependent ubiquitination of p53 is regulated by hypoxia.
The p53 pathway is activated under hypoxic conditions. Therefore, we investigated whether SUMOylation would play a role in the p53 response to hypoxia. Hypoxia has been reported to regulate other proteins via SUMOylation, such as the Hypoxia-Induced Factors 1 and 2. Stable MCF-7/His6-SUMO-2 cells were used to study the effects of hypoxia on the ubiquitination of SUMOylated p53, Hdm2 and Hdmx. As expected, we observed a strong induction of HIF1-alpha and some increase in p53 protein levels after 48 hours of hypoxia (1% O2), which was absent in cells that were treated with MG132. On the other hand, Hdm2 and Hdmx levels were reduced upon hypoxia. This reduction could not be rescued by inhibition of the proteasome, suggesting that it is not mediated by increased protein degradation (Fig. 5A). Furthermore, we observed a decrease of p53, Hdm2 and Hdmx SUMOylation and ubiquitination of the SUMOylated proteins (Fig. 5B). The observed decrease in Hdm2 and Hdmx SUMOylation could very well be explained by the decreased total protein levels, indicating that the relative SUMOylation is not affected. However, the increase in p53 total protein levels might be the result of reduced p53 SUMOylation and ubiquitination under hypoxic conditions. These results indicate that hypoxia leads to reduced mRNA expression of Hdm2 and Hdmx, but does not influence the SUMOylation status of these proteins. In contrast, SUMOylation and ubiquitination of p53 are reduced under hypoxic conditions, thereby stabilizing the protein.

SUMOylation of p53 is reduced and SUMOylated Hdm2 is degraded upon DNA damage.
To find out more about the role and regulation of SUMOylation in the activation of the p53 pathway after stress, we treated cells with various types of DNA damaging agents. His6-SUMO-2 expressing MCF-7 cells were treated with the proteasomal inhibitor MG132 with or without etoposide, or mock-treated. The relatively unstable p53 protein is strongly stabilized by MG132 treatment and also in response to etoposide (Fig. 5C). Confirming the results presented in figure 1, the relative amount of SUMOylated p53 does not increase upon proteasome inhibition. Etoposide treatment leads to an increase in the SUMOylated...
p53 fraction in absence of MG132, reflecting the increase in total p53 levels. However, MG132 treatment upon etoposide incubation results in a decreased accumulation of high molecular SUMOylated/ubiquitinated p53 species, suggesting that p53 SUMOylation is reduced after DNA damage, similar to what is observed under hypoxic conditions. In contrast, the amount of the relatively stable protein Hdmx is not significantly affected by proteasomal inhibition up to 6 hours, but Hdmx protein levels are strongly decreased upon etoposide treatment via Hdm2-mediated ubiquitination. The SUMOylated Hdmx fraction is increased when the proteasome is inactivated, indicating a rapid degradation of SUMOylated Hdmx species, as already presented in Figure 1. Upon treatment with etoposide, the relative amount of SUMOylated Hdmx appears to be slightly increased, both noticed in presence and absence of MG132 treatment (Fig. 5C). Treatment of the cells with the DNA damaging agent NCS resulted in a similar effect on both total and SUMOylated Hdmx (Fig. 5D). These results suggest that upon DNA damage, Hdmx is degraded in a SUMO-independent way.

Interestingly, the SUMOylated fraction of Hdm2 is decreased upon NCS treatment, whereas after combined NCS and MG132 treatment a strong increase of ubiquitinated SUMOylated Hdm2 species is observed (Fig 5D). This result suggests that DNA damage stimulates the ubiquitination and degradation of SUMOylated Hdm2, whereas total Hdm2 levels actually increase, due to increased p53-mediated transcription of the Hdm2 gene.

Role of RNF4 in the DNA damage response.

RNF4 and its yeast counterparts Slx8/Rfp are involved in the DNA repair response upon DNA damage and are essential for genomic stability; cells lacking RNF4 or its yeast counterparts are suggested to be more sensitive for DNA damage. It has been suggested RNF4 plays a role in DNA repair mechanisms via demethylation of the DNA, for which both its SIM and RING domain are required. Notably, PML, an RNF4 target, is a negative regulator of DNA repair genes and its RNF4-mediated degradation might enhance DNA repair. Upon arsenic trioxide (ATO) treatment, PML is degraded via RNF4-mediated poly-SUMO-dependent ubiquitination. Furthermore, ATO treatment has been shown to induce ATR, resulting in activation of CHK2 and p53. Indeed, ATO treatment of U2OS cells for 24 or 48 hrs resulted in induction and activation of p53 and p53 targets, such as the cell cycle inhibitor p21 and the pro-apoptotic PUMA, and in decreased PML levels (Fig. 5E). Surprisingly, RNF4 levels were also decreased after ATO treatment (Fig. 5E). Interestingly, the amount of high molecular species of SUMOylated p53, Hdm2 and Hdmx was also reduced upon ATO treatment, also when the proteasome is inhibited (Fig. 5F), whereas total protein levels were not reduced. These results suggest a decrease in SUMO-specific
ubiquitination of certain SUMOylated proteins after ATO treatment, which might be associated, or at least coincide, with the observed decrease in RNF4 levels.

**RNF4 degradation correlates with induction of apoptosis.**

The degradation of RNF4, as seen after ATO incubation, was also observed upon various other treatments, including UV-C, cisplatin, Nutlin-3, IR and RITA (Fig 6A and B), which all induce a p53 response. The small molecule RITA has been reported to induce apoptosis in tumor cells, in a p53-dependent way, and elicits a DNA damage response \(^{71,72}\). Interestingly, in contrast to wild-type HCT116 cells, no decrease of RNF4 levels was found in p53-/- HCT116 or Chk2-negative HCT116 cells when treated with RITA (Fig. 6B), suggesting that a p53-Chk2 pathway is required in this process. Both p53-/- and Chk2-/- HCT116 cells are very resistant to RITA-induced apoptosis \(^{71}\) and De Lange et al., personal communication), suggesting a correlation between induction of apoptosis by RITA and decrease in RNF4 levels. The p53-dependency of the observed RNF4 degradation might be depending on cell-type or drug concentrations, since a similar RNF4 decrease in U2OS control and U2OS p53-knockdown cells upon ATO treatment is observed (Fig. 6C).

Confirming a role for RNF4 in the response to certain drugs, MCF-7 cells that overexpressed Flag-tagged RNF4 were more resistant to RITA-treatment than control cells in short-term growth assay (Table 1 and Fig. 6D).

### Table 1. Effective Dose of RITA treatment on MCF7 and HCT116 cells expressing a control- or an RNF4 expression vector.

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MCF7 and HCT116 cells were lentivirally infected with a control- (pLV-mCherry-GFP) or a Flag-RNF4-GFP expression vector. At day 4 after infection, cells were seeded for WST-1 proliferation assays and cells were mock treated or treated with a range of concentrations of RITA. Doses used for MCF7 cells were 10, 20, 40 and 80 nM RITA, whereas HCT116 were treated with 100, 200, 400 and 800 nM RITA, all for 24 hours. Medium was replaced with fresh medium without RITA, and relative survival was measured 72 hrs later and effective dose-curves were calculated using the CompuSyn program. The effective dose for the indicated Affected Fractions (Fa) was calculated using these curves.
Figure 5. Regulation of SUMOylation in the p53-pathway by stress.
MCF7 cells stably expressing His6-SUMO-2 were incubated at hypoxic (1% O_2) conditions, or maintained at normoxia (20% O_2) for 48 hours, followed by treatment with 10 µM MG132 or mock treated for 6 hours. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates (A) and His6-SUMO-2 purified fractions (B) were analyzed by immunoblotting with the indicated antibodies. (A,B) MCF7 cells stably expressing His6-SUMO-2 were either mock treated (-), treated with 10 µM MG132 for 1 hour (+) or 6 hours (++) and treated with 10 µM etoposide (A) or 500 ng/ml NCS (B) for 6 hours, with or without the addition of MG132, 15 min before drug treatment, as indicated. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 purified fractions were analyzed by immunoblotting with the indicated antibodies. (C) U2OS cells were treated with 3 µM arsenic trioxide (ATO) or mock-treated for 24 and 48 hours. Cells were lysed; protein extracts were analyzed by immunoblotting with the indicated antibodies. (D) MCF7 cells stably expressing His6-SUMO-2 were either mock-treated or treated with 3 µM arsenic trioxide for 6 and 24 hours; 6 hours before harvesting 10 µM MG132 was added. Cells were lysed and His6-SUMO-2 conjugates were purified using Ni-NTA beads. Total cell lysates and His6-SUMO-2 purified fractions were analyzed by immunoblotting with the indicated antibodies. Vinculin expression was analyzed as a loading control.
Figure 6. Functions of RNF4 in the apoptotic response upon various drug treatments.

(A) MCF7 cells were irradiated with UV-C (10 J/m²) or mock treated, treated with 5 µM cisplatin or treated with 10 µM Nutlin-3. HCT116/p53+/+ were mock treated or irradiated with ionizing radiation (IR; 10 Gy). Cells were harvested at the indicated times after start of treatment, protein extracts were analyzed by immunoblotting with the indicated antibodies. (B, upper panel) HCT116/p53+/+, HCT116/p53-/- and HCT116/Chk2-/- cells were treated with 1 µM RITA or mock-treated for 24 hours; protein extracts were analyzed by immunoblotting with the indicated antibodies, USP7 expression was analyzed as a loading control. (B, lower panel) HCT116 p53+/+ and HCT116 p53-/- cells were treated with 1 µM RITA for the indicated times. Protein extracts were analyzed by immunoblotting with the indicated antibodies. Tubulin expression was analyzed as a loading control. (C) U2OS cells were lentivirally infected with either an expression vector encoding a non-coding shRNA or a shRNA against p53. Four days after transduction, cells were treated with the indicated concentrations ATO for 24 hours.
RNF4 and SUMOylation in the p53 pathway

were harvested and protein extracts were analyzed by immunoblotting with the indicated antibodies. USP7 expression was analyzed as a loading control.

(D) MCF7 cells were lentivirally infected with an empty control vector or a vector encoding Flag-RNF4 or RNF4-Flag. Four days after transduction, cells were seeded for WST-1 proliferation assays, colony formation assays and protein analysis. For WST-1 proliferation assays cells were seeded at a density of 2500 cells per well. One day after seeding, cells were treated with the indicated concentrations of RITA for 24 hours or mock-treated. Relative survival was determined at day 3 after treatment. (E, F) Cells were seeded for colony formation assays in 6-well plates with a density of 10,000 cells per well and treated with 50 nM RITA for 3 hours, or irradiated with IR with the indicated doses, one day after seeding. One week later colonies were quantified and displayed as relative colony formation compared to the corresponding mock-treated cells. (G) For analysis of protein expression levels, cells were treated with 160 nM RITA for 24 hours or mock-treated, one day after seeding. Cells were harvested and protein extracts were analyzed by immunoblotting with the indicated antibodies. USP7 expression was analyzed as a loading control. A-specific bands detected with the anti-RNF4-antibody are indicated with *.

Moreover, long-term colony formation assays also indicated that cells with increased RNF4 levels were more resistant to RITA treatment (Fig. 6E). In contrast, no differences were found when the same cells were treated with ionizing radiation, NCS or topotecan (Fig. 6F and data not shown).

In the RNF4-overexpressing MCF7 cells, protein analysis showed no differences in p53 phosphorylation on Ser15, no differences in Hdm2 induction, but reduced PARP cleavage, an indicator for apoptosis, after treatment with RITA (Fig. 6G). However, the effect of RNF4 overexpression appears to be cell-type and/or dose dependent, since overexpression of RNF4 in HCT116 cells only very slightly increased resistance of RITA-induced growth inhibition/ apoptosis, and did not significantly affect PARP cleavage (Table 1 and Fig. 6H). On the other hand, HCT116 cells are much more resistant for RITA-induced apoptosis than MCF7 cells, so a comparison between these cell lines is difficult to make.
Chapter 6

Discussion

Key proteins in the p53 pathway are strongly regulated by post-translational modifications, such as phosphorylation, acetylation, ubiquitination and SUMOylation. The function of the latter modification is not very well understood yet; it might fulfill roles in localization, activity and degradation. Evidence exists for several ways of cross-talk between SUMO and ubiquitin \(^{73,74}\), which may have different outcomes, varying from protein stabilization to degradation, depending on the type of interaction. The SUMOylation of p53 and Hdm2 have been reported to inhibit transcriptional activity \(^{29}\) and auto-ubiquitination \(^{42}\), respectively. SUMOylation of Hdmx has not been reported to have a distinct function yet, as a SUMO-mutant Hdmx did not respond differently from wild-type Hdmx regarding Hdm2-mediated degradation, subcellular localization, DNA damage response and p53 inhibition \(^{38}\).

In this study we investigated the regulation of the SUMOylation of p53, Hdm2 and Hdmx and observed that under certain circumstances the SUMOylated proteins were preferentially ubiquitinated and degraded. This phenomenon is especially clear with regard to Hdmx; proteasome inhibition hardly affects total Hdmx levels, but a strong accumulation of high molecular weight species of the SUMO-conjugated Hdmx protein is observed, most likely representing poly-ubiquitinated Hdmx species. These findings indicate a role for SUMOylation and SUMO-dependent ubiquitination in the regulation of p53. We found that inactivation of ERK1/2 reduces the ubiquitination of SUMOylated p53, Hdm2 and Hdmx, whereas Hdm2 protein expression is also reduced, most likely at the mRNA level. Consistent with a previous published observation that SUMOylation inhibits p53 activity \(^{29}\), we observed a reduced p53 SUMOylation and ubiquitination of SUMOylated p53 after p53 activation by various stresses, such as hypoxia and DNA damage. SUMOylation of Hdm2 is reduced after stress, most likely due to increased degradation of SUMOylated Hdm2, whereas Hdmx seems to be degraded in a SUMO-independent way.

A putative candidate E3 ligase for SUMO-dependent ubiquitination of the proteins in the p53 pathway is the STUbL RNF4. RNF4 has been reported to specifically ubiquitinate poly-SUMOylated PML, but no other targets have been identified to date. If SUMOylated p53, Hdm2 and Hdmx are also targets for RNF4-mediated ubiquitination, RNF4 knockdown should reduce the ubiquitination of SUMOylated proteins, resulting in stabilization of the SUMOylated species. However, upon RNF4 knockdown we actually observed a decrease in both SUMOylated and ubiquitinated p53, Hdm2 and Hdmx.
Moreover, also total protein levels of Hdm2 and Hdmx are somewhat decreased, whereas p53 levels are not much affected, or slightly induced in RNF4 knockdown cells. Overexpression of RNF4 seems to stabilize Hdm2, suggesting its main activity on Hdm2 is SUMO-independent or an indirect interaction.

Upon DNA damage, p53 is activated, resulting in either cell cycle arrest, to enable the repair of damaged DNA, or apoptosis, when the damage is not repairable.

RNF4 can be associated in several ways with the DNA damage response and thereby possibly with the p53 pathway. First of all, RNF4 has been shown to have a role in DNA demethylation, involving the Base Excision Repair (BER) pathway. Consistently, RNF4 knockout MEFs showed a strongly increased accumulation of DNA damage during cell culture. Importantly, there is evidence that p53 might regulate this DNA repair pathway; therefore, it is not excluded that RNF4 might influence the DNA repair system via the p53 pathway. Another possibility is that RNF4 affects the p53 pathway indirectly via PML. It has been reported that PML enhances the p53 activation upon DNA damage by recruiting p53 and several kinases to the PML bodies, resulting in phosphorylation of p53 on Thr18 and Ser20, which protects from Hdm2 inhibition. Upon arsenic trioxide treatment, the SUMOylation of PML is greatly enhanced, followed by degradation by RNF4. Although the degradation of PML is only seen upon treatment with arsenic trioxide, the control of PML by RNF4 under normal conditions or in response to other drugs cannot be excluded and might have a function in the regulation of the p53 pathway. Furthermore, it has been shown that PML interacts with Hdm2, mediating its localization to the nucleoli after DNA damage. The activity of RNF4 on PML may therefore also have an effect on Hdm2 stability. Not much is known about the interaction between Hdmx and PML, but it is well possible that Hdmx is also regulated by PML.

Overexpression of RNF4 stabilized the Hdm2 protein, which makes a role of RNF4 in the regulation of the p53 response feasible. We found that RNF4 levels decrease upon various drugs treatment. In the case of RITA, the downregulation of RNF4 correlates with activation of p53 and an apoptotic response, which was found to be absent in p53 and Chk2-negative HCT116 cells. Interestingly, in some cases the downregulation of RNF4 is concomittant with the activation of p53, and is, for example, also observed after treatment with the p53 activator Nutlin-3. However, activation of p53 is not sufficient for RNF4 downregulation since treatment of MCF-7 cells with etoposide or topotecan is activating p53 but does not or hardly affect RNF4 levels. In addition, downregulation of RNF4 by ATO treatment is similar in control and p53-knockdown U2OS cells.
Nevertheless, elevated RNF4 levels in MCF7 cells attenuated the apoptotic response upon RITA treatment, indicating that the degradation of RNF4 might facilitate an appropriate apoptotic response, which might well be p53-independent. It would, therefore, be useful to investigate the response to apoptosis-inducing drugs in cells that have reduced RNF4 levels, as it may be expected that these cells will be more sensitive for such drugs. Preliminary results indicate that RNF4-knockdown cells show a strong growth inhibition upon a few passages and, therefore, the analysis of drug responses is complicated. It could be informative to screen a large set of tumor and normal cells for a putative correlation between RNF4 expression levels and the induction of apoptosis.

More insight into the mechanism by which RNF4 is degraded to allow an apoptotic response might serve as a potential lead to development of new anti-cancer drugs, since reducing RNF4 likely will enhance the killing effect of conventional anti-cancer treatments. On the other hand, reduced RNF4 levels were found to be associated with testicular tumor progression, due to a growth inhibiting function in this specific cell-type \textsuperscript{79-81}. Interestingly, these tumor cells are in general very sensitive to chemotherapeutic treatments \textsuperscript{82}. Even so, it is not excluded that overexpression of RNF4 may act as an oncogene in other tissues. Enhanced RNF4 expression was found to be associated with tumor formation in BALB-neuT mammary glands and found to be enhanced in breast tumors \textsuperscript{83}. These results would be consistent with our results; overexpression of RNF4 might also inhibit oncogene-mediated p53 activation or apoptosis, thereby promoting tumourigenic transformation.

Acknowledgments:

We thank Alfred Vertegaal, Ivo Hendriks and Joost Schimmel for helpful discussions regarding the RNF4 project and the gift of RNF4-expression vectors, RNF4 antibody and MCF-7 cells stably expressing Flag-RNF4 or RNF4-Flag.
References

Chapter 6

RNF4 and SUMOylation in the p53 pathway

## Supplemental Information

### q-RT-PCR primer sequences

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<th>Primer</th>
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FAU expression is essential for proliferation and affects the drug sensitivity of osteosarcoma cells.

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Chapter 7

Abstract

The \textit{FAU} gene (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-Associated Ubiquitously expressed gene) was originally identified as a pro-apoptotic gene and its downregulation has been associated with tumourigenicity and drug resistance in tumour cell lines of various origins. The \textit{FAU} mRNA encodes for a fusion product of the ubiquitin-like protein FUBI and the ribosomal protein S30, which is cleaved after a Gly-Gly motif, yielding the mature proteins. It has been demonstrated that the FUBI protein can be conjugated to the pro-apoptotic Bcl-2 family member Bcl-G, which was reported to be essential for the induction of apoptosis by FAU. In contrast, others have reported that FAU and FUBI overexpression has transforming capabilities, whereas FAU and S30 overexpression conferred arsenite resistance. In this study we investigated the function of the \textit{FAU} gene in osteosarcoma and found that \textit{FAU} expression is essential for cell proliferation. Knockdown of \textit{FAU} resulted in a strong decrease of the proliferation rate caused by a G2/M arrest, accompanied by an increase of the checkpoint protein GADD45-alpha. This effect could only be rescued by the ectopic overexpression of the complete \textit{FAU} mRNA, suggesting an essential function for both FUBI and S30. Furthermore, we demonstrated that cells with reduced \textit{FAU} expression showed less sensitivity to treatment with various types of chemotherapeutical drugs, confirming a role of FAU in determining drug sensitivity.
Effects of FAU expression in osteosarcoma cells

Introduction

Ubiquitination is an important post-translation protein modification involved in the regulation of many cellular processes. Ubiquitin is conjugated to a target protein by formation of an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine in the target protein. A target protein can be (multiple) mono-ubiquitinated or poly-ubiquitinated. Dependent on the ubiquitination status, ubiquitination may lead to altered localization or function of the protein, or can trigger the 26S-proteasome-mediated degradation of the target protein, reviewed recently \(^1\). A number of ubiquitin-like modifiers have been identified, like SUMO and NEDD8 \(^3\), the functions of which are being gradually unraveled, reviewed in Gareau and Lima (2010) \(^4\) and Watson et al. (2011) \(^5\). One of the least studied ubiquitin-like proteins is FUBI, which is translated as a fusion protein named FAU. The FAU gene encodes a 133 amino acid fusion protein, consisting of the N-terminal ubiquitin-like FUBI protein (74 amino acids) and the C-terminal ribosomal protein S30 (59 amino acids). The FUBI protein contains a C-terminal di-glycine motif, like ubiquitin, enabling it to be conjugated to target proteins. The FUBI protein is \(\sim 35\%\) identical to ubiquitin, but, strikingly, lacks almost all internal lysines with the exception of lysine 25, corresponding to lysine 27 in ubiquitin. Not much is known about the function of K27 linkage in ubiquitin, although it has been suggested that all ubiquitin linkages can target proteins for proteasomal degradation \(^6\). K27 linkage was reported to be assembled by U-box-type 3 E3 ligases under certain stress responses \(^7\), or by TRAF6 overexpression \(^8\). Furthermore, the RingB1 E3 ligase promotes self-ubiquitination using mixed a-typical ubiquitin chains, including K27, which are required for its ligase activity towards Histone H2A \(^9\). Interestingly, the FUBI (Ubi-L) domain of FAU has been reported to bind non-covalently to Histone H2A \(^10\).

The FAU gene (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-Associated Ubiquitously expressed gene) was identified in two independent studies by functional expression cloning to be a novel apoptosis regulator. Originally, the FAU gene was identified as the cellular homologue of the fox sequence in FBR-MuSV, isolated from a radiation-induced mouse osteosarcoma \(^11\). The viral fox sequence is expressed as an antisense of the mouse FAU cDNA sequence and was found to posses transforming capabilities, most likely by inactivating endogenous FAU expression \(^12\). The FAU gene is conserved in higher eukaryotes and expressed in all mammalian tissues \(^12,13\).

The FAU antisense sequence inferred resistance to cisplatin and UV-radiation in mouse T-cells. Moreover, overexpression of FAU was shown to induce apoptosis in several cell
Chapter 7

lines, revealing a pro-apoptotic function. In these studies the putative distinct functions of FUBI and S30 were not addressed. A possible explanation for the role of FUBI in regulation of apoptosis was initially provided by Nakamura et al. (2003). They showed that the FUBI domain of FAU, also called MNSFbeta, could be conjugated to the pro-apoptotic Bcl-2 family member Bcl-G, thereby inhibiting the ERK cascade. In subsequent studies it was shown that regulation of Bcl-G by FUBI modification is essential for its pro-apoptotic activity, and that FAU expression is frequently downregulated in breast cancer development, which was associated with poor patient survival. Selection for low FAU expression was also observed in ovarian and prostate cancer, correlating with reduced Bcl-G expression. Furthermore, reduction of FAU expression in the A2780 ovarian cancer cell line conferred some carboplatin resistance to these cells. In line with these results, the downregulation of FAU and Bcl-G expression in the prostate cancer cell line 22Rv1 as well as the breast cancer cell line T-47D resulted in decreased sensitivity to UV-C irradiation. These results were recently repeated in a study containing three more human cell lines, and it was shown that FAU overexpression somewhat decreased the number of viable cells. All these studies indicate a tumour suppressor role for FAU in cancer development.

In contrast, in another study, the FAU sequence was shown to protect against arsenic-induced apoptosis. The same authors later showed that overexpression of FAU or its FUBI domain could transform the osteosarcoma cell line HOS to anchorage-independence, whereas the FAU or the S30 subunit alone both induced arsenite resistance. These results would suggest an oncogenic function for FAU.

Furthermore, it was demonstrated that the FAU ubiquitin-like domain is involved in the immune response, although this might be independent from its pro-apoptotic function.

We investigated the role of FAU in the sensitivity of osteosarcoma cell lines to several types of drugs, including arsenic trioxide (ATO). Arsenic trioxide is used to induce apoptosis in acute promyelocytic leukemia (APL) and was also reported to induce apoptosis in solid cancer cell lines. Furthermore, it was shown that arsenic trioxide treatment induced apoptosis via p73, which could be enhanced with MEK1 inhibitors. Recently, it has been reported that ATO treatment reduced motility, migration and invasiveness in the osteosarcoma cell lines HOS and MNNG, probably by the inhibition of phosphorylation of MAPK family members ERK and MEK. Several groups report that in other osteosarcoma cell lines, such as MG-63 or SaOS-2, ATO treatment successfully can induce apoptosis and act synergistically with other drugs.
Effects of FAU expression in osteosarcoma cells

On the other hand, exposure to low concentrations of ATO has been suggested to act carcinogenic \(^{34-36}\), possibly by the observed activation of the p38-MAPK, ERK1/2 and Akt pathways upon low dose treatment with ATO \(^{37}\).

To investigate FAU functions on drug sensitivity, we performed both knockdown of \(FAU\) and overexpression of FAU, FUBI or S30 and assessed the effects of drug treatments. Surprisingly, we found a significant growth inhibiting effect of \(FAU\) knockdown, which could only be rescued by concomitant overexpression of the whole FAU sequence. Furthermore, we observed decreased sensitivity for arsenic trioxide, cisplatin and doxorubicin in cells with decreased FAU levels. On the other hand, overexpression of FAU and FUBI increased the sensitivity for these drugs.

Material & Methods

Cell Culture and Reagents
U2OS, OST and OSA cells were maintained in RPMI supplemented with 10% FBS and antibiotics. Cells were treated with arsenic trioxide, doxorubicin, cisplatin, Actinomycin D (all from Sigma-Aldrich, St Louis, MO, USA), MEK1/2 inhibitor U0126 (Calbiochem), MEK1 inhibitor PD98059 (Calbiochem), RITA (Gift from Galina Selivanova) and Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA) for the indicated times and concentrations.

Plasmids, Lentiviral Transduction
A \(FAU\) cDNA clone was obtained from Origene Technologies Inc. (Rockville, MD, USA). The coding sequences of \(FAU\), \(FUBI\) or \(S30\) were amplified, with the use of 5’ primers containing a BamHI and 3’ primers containing an XbaI restriction site. The resulting fragment was cloned into a pcDNA3.1 expression vector containing an HA-tag such that the HA-tag is N-terminal of the \(FAU\), \(FUBI\) or \(S30\) coding region. Lentiviral expression vectors for HA-FAU, HA-FUBI and HA-S30 were made by cloning the corresponding cDNA sequences into pLV-CMV-bc-puro using \(NdeI\) and \(NheI\) restriction sites. cDNA sequences are available in supplemental information.

For \(FAU\) knockdown 2 different lentiviral vectors containing a short hairpin RNA targeting \(FAU\) are used (Sigma MISSION Library; Sigma-Aldrich). Targeting sequences are: shFAU #1: 5’- CAGACTCCTAAGGTGGCCAAA-3’ and shFAU#2: 5’- GAAGAAGACAGGTCGGGCTAA-3’. These shRNAs target the \(S30\) coding region with the \(FAU\) sequence; unfortunately the other shRNAs targeting \(FAU\) within the \(FUBI\) coding region were very inefficient in downregulating \(FAU\) mRNA (not shown).

Other lentiviral shRNA expression vectors were the non-targeting shRNA expression lentivirus shc002 (Sigma MISSION Library; Sigma-Aldrich), shRNA targeting human \(p53\) \(^{38}\) or mouse \(mdmX\) \(^{39}\) as a control. The construction of lentiviral vectors expressing specific shRNAs and the production of lentivirus particles have been described recently \(^{39}\).

Proliferation assay, Colony Forming assay, FACS analysis
For growth assays, 2000 cells were seeded in triplicate in a 96-wells plate; 24 hours after seeding, cells were treated with the indicated drugs for the indicated times. To measure cell survival, cells were incubated with WST-1 reagent (Roche Biochemicals, Indianapolis, IN) for 1-2 hours followed by quantification with a plate reader.
RNA isolation, Reverse Transcription, Q-RT-PCR
RNA was isolated from cells using the SV Total RNA Isolation System (Promega) according to the manufacturers’ protocol. cDNA was made from 1 µg of total RNA in a Reverse Transcriptase reaction containing ImPromII Reverse Transcriptase, RNasin RNase inhibitor, MgCl2; and 5x RT-Buffer (Promega). Quantification of mRNA expression was performed by Quantitative-real-time PCR, in an ABI 7900HT thermocycler, using the FastStart Universal SYBR Green Master (ROX) mix (Roche). Relative mRNA quantities were calculated using an internal standard curve, made from a dilution series of the pooled samples, using the SDS2.3 analysis program (Applied Biosystems). Expression levels of the mRNAs were normalized to the geometric mean of the control genes CAPNS1, TBP, SRPR. Primer sequences are available in Supplemental Information.

Protein extraction, Western Blotting and Antibodies
Cells were lysed in Giordano 250 buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA), supplemented with a mixture of protease- and phosphatase inhibitors. Protein content was determined using Bradford Reagent (Biorad) and equal amounts of protein were separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Membranes were blocked in Tris-buffered saline, 0.2% Tween-20 (TBST) containing 10% non-fat dry milk, and subsequently incubated with the appropriate primary antibody diluted in TBST containing 10% milk. Membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibody (Jackson Laboratories). Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce) followed by detection with the ChemiGenius XE3 system (Syngene, Cambridge, UK) or X-ray film (Fuji).
Antibodies used in this study were anti-HA.11 monoclonal mouse antibody (Covance), rabbit polyclonal anti-USP7 (A300-033A, Bethyl Laboratories), anti-S30 rabbit polyclonal antibody (405, gift of Dr. J.C. Marine), anti-B23 mouse monoclonal antibody NA24 (Santa Cruz), anti-phospho-ERK1/2 monoclonal mouse antibody (Sigma), anti-ERK1/2 rabbit polyclonal antibody (Sigma), anti-phospho-Akt-S473 rabbit polyclonal antibody (Cell Signaling), anti-Akt1/2 rabbit polyclonal antibody (Upstate), anti-phospho-T246 PRAS40 rabbit polyclonal antibody (Cell Signaling), anti-cyclin D1 rabbit polyclonal antibody (Santa Cruz) and anti-p27 rabbit monoclonal antibody (Epitomics). Secondary antibodies goat-anti-mouse-HRP and goat-anti-rabbit-HRP were obtained from Jackson Laboratories.

Immunofluorescence:
Cells were seeded onto coverslips 24 hours pre-fixation. Cells were washed with PBS before fixation with 4% paraformaldehyde for 10 min at room temperature (RT). Cells were subsequently washed with PBS, permeabilized in PBS with 0.2% Triton X-100 solution for 10 min at RT, washed with PBS and washed with PBS containing 0.05% Tween-20. Cells were blocked with PBS containing 0.05% Tween-20 and 5% Normal Goat Serum (NGS) for 1 hour at RT, incubated with the indicated primary antibodies, diluted in PBS/Tween-20/NGS, for 1.5 hours. After washing, cells were incubated for 30 min with goat anti-mouse-Rhodamine secondary antibody (Jackson) diluted 1:100 in PBS/Tween20/NGS. After washing, coverslips were mounted onto microscope slides using DAPI-DABCO mounting solution, containing 90% glycerol, 10% 0.2M Tris-HCl pH8.0, 2.3% DABCO (1,4 Diazabicyclo [2,2,2] octane) and 0.002% DAPI.
Results

**FAU expression is essential for cell proliferation**

To assess a putative role of FAU expression in drug sensitivity of osteosarcoma cell lines, we performed both *FAU* knockdown- and overexpression experiments. Initially, we performed a knockdown of *FAU*, with 2 different lentiviral knockdown constructs, in 3 osteosarcoma cell lines, U2OS, OST and OSA, which have slightly varying basal *FAU* mRNA levels (Fig. 1A). Knockdown resulted in at least a 50% decrease in *FAU* mRNA levels (Fig. 1A). Strikingly, in all three cell lines, knockdown of *FAU* resulted in a marked growth inhibition with both knockdown vectors (Fig. 1B and C).

To investigate whether the observed inhibition of growth is indeed caused by the reduced *FAU* expression, we tried to rescue the knockdown effect by overexpression of FAU, FUBI or S30. To that end, U2OS cells were transduced with lentiviral vectors expressing HA-FAU, HA-FUBI or HA-S30 or a control vector, and stably transduced cells were obtained by puromycin selection. Subcellular localization of the exogenously expressed proteins was investigated with by immunofluorescence. HA-FAU was found to be present in the nucleus, including the nucleoli (Suppl. Fig. 1A). HA-S30 was predominantly localized in the nucleoli, as demonstrated by co-localization with the nucleolus-specific protein B23 (Suppl. Fig.1B). HA-FUBI was localized throughout the cell, both nuclear and cytoplasmic; however, patterns were strongly variable and might be determined by e.g. cell cycle phase. (Suppl. Fig. 1A). Not all cells transduced with HA-FUBI or HA-S30 lentiviruses detectably expressed the corresponding protein as determined by immunofluorescence, whereas virtually all HA-FAU transduced cells do express HA-FAU. Expression of endogenous FAU, FUBI or S30 was not detectable with the available antibodies; neither by immunofluorescence nor by western blotting.

Subsequently, *FAU* was knocked down with the same lentiviral knockdown constructs mentioned above, which target endogenous *FAU* mRNA in its coding region, in the *S30* part of the sequence. Consequently, exogenous *FAU* and *S30* will also be targeted for shRNA-mediated degradation upon transduction with these knockdown constructs, whereas exogenous *FUBI* should not be affected.

The *FAU* knockdown efficiency in all 4 stable U2OS cell lines is shown in Supplemental Figure 1C. Depicted are the relative mRNA quantities determined with the use of primer sets for respectively endogenous *FAU*, total *FUBI* and total *S30*. At the mRNA level, both *FAU* and *FUBI* are highly expressed, whereas *S30* is somewhat lower. Both *FAU* and *S30* are decreased upon *FAU* knockdown, whereas *FUBI* mRNA levels remain constant. Nevertheless, compared to control cells, the *FAU* overexpressing cells still have much
higher FAU mRNA levels after FAU knockdown, and even S30 overexpressing cells still retain about 5-10x higher S30 mRNA levels compared to control after knockdown of FAU.

At the protein level (Suppl. Fig. 1D) both HA-FAU and HA-S30 expression are decreased upon FAU knockdown, although the HA-FAU expression is still well detectable. HA-FUBI expression is not significantly affected, as expected. In the HA-FAU cells we also detect a band of the same size as HA-FUBI. This most likely indicates that FAU is cleaved at the GG-motif, which essentially yields an HA-FUBI protein (detected with anti-HA antibody) and the untagged S30 protein, which is not detected. Interestingly, a smear of high molecular species is detected in both HA-FUBI and HA-FAU expressing cells, which most likely consists of FUBI-conjugates. Since S30 is lower expressed, a longer exposure of the blot is shown. Probably therefore some aspecific bands were detected in both Ctrl and S30 cells, but the intensity is much lower than the FUBI-conjugates detected in the FUBI and FAU cells.

Immunofluorescence on cells that had FAU knocked down, confirmed the results found on western and RNA; HA-FAU overexpressing cells showed a slightly decreased signal for FAU and nucleolar staining was more obvious. HA-FUBI expression and localization seemed to be unaffected, whereas the specific S30 signal in HA-S30 cells was significantly reduced (Suppl. Fig. 1A).

Although FAU has been reported to be a pro-apoptotic gene and, therefore, a growth reduction might be expected, overexpression of both FAU and FUBI did not significantly change the growth rate of the cells, whereas cells with S30 overexpression showed a somewhat reduced proliferation rate (Fig. 1D).

In contrast, knockdown of FAU resulted in growth inhibition in control-, HA-FUBI- and HA-S30 overexpressing cells, but was almost completely rescued by HA-FAU overexpression (Fig. 1E). Furthermore, we also observed partial rescue of the growth inhibitory effect upon FAU knockdown by HA-FAU overexpression in a long term growth assay, a colony forming assay (Fig. 1F).

FACS analysis was performed to find an explanation for the growth-inhibition seen upon knockdown of FAU (Suppl. Fig. 2A). The most striking change observed is a strong increase of the number of cells in G2/M phase upon FAU knockdown, with concomitant reduced G1- and S-phase cells. Furthermore, a slight increase of cells in SubG1 is observed, suggesting some induction of apoptosis. Overexpression of FAU, FUBI or S30 did not significantly change the cell cycle profile (Suppl. Fig. 2A). Importantly, HA-FAU overexpression largely rescued the cell cycle effects seen upon FAU knockdown. The constitutive expression of FUBI or S30 could not or only slightly rescue the cell cycle effects. However, since particularly the levels of HA-S30 are lower than the HA-FAU
Figure 1. FAU expression is essential for cell proliferation

U2OS, OST and OSA cells were lentivirally transduced with control-, or two different FAU knockdown constructs. Cells were seeded for proliferation assays and RNA isolation 24 hours after infection. RNA extracts were isolated at day 4 post-infection. Expression levels of FAU mRNA were quantified using q-RT-PCR and are
shown as normalized relative mRNA levels (A). Metabolic activity as a measure of proliferation was measured at the indicated days and displayed as relative growth to day 0 (B) or as relative survival at day 2 compared to control cells (C). U2OS cells were lentivirally transduced with either control vector or expression vectors for HA-FUBI, HA-S30 or HA-FAU. Stable cell lines were established by puromycin selection and survival relative to control cells was measured 3 days after seeding using WST-1 proliferation assays (D). Stable cell lines were lentivirally infected with either control or two different FAU knockdown constructs. Cells were seeded for WST-1 proliferation assays and colony formation assays 48 hours after infection. Relative survival of transduced cells was measured 3 days after seeding (E). Colony formation of stable control or HA-FAU expressing cells transduced with either control or FAU knockdown was quantified 10 days after seeding and displayed as relative colony formation compared to the corresponding control cells (F).

expression after FAU knockdown, the rescue effect might be attenuated compared to the effect of FAU overexpression. However, based on the observations in short and long term proliferation assays and the results of flow-cytometry, it seems that the expression of the complete FAU sequence is needed to rescue the growth inhibiting effect of FAU knockdown.

**FAU expression enhances sensitivity for ATO treatment in U2OS cells.**

To assess a putative effect of FAU levels on the sensitivity to arsenic trioxide (ATO) in osteosarcoma cells, we treated the above described stable cell lines for 24 hours with 3 µM ATO and cell survival was determined. Although the control cells show a relative survival of only 25% after 72 hrs, cells that overexpress FAU show an even lower relative survival (Fig. 2A). Moreover, as illustrated in the growth curves (Fig. 2B), ATO-treated control cells are just growth arrested, whereas the number of ATO-treated FAU overexpressing cells actually decreases, suggesting cell loss through apoptosis. Furthermore, cells with FAU knockdown, which already have a somewhat reduced growth rate, show almost no further reduction upon ATO treatment (Fig. 2C). Cells that have FAU overexpression and FAU knockdown combined, show an intermediate survival (Fig. 2B). FACS analysis showed an increased number of control cells in G2/M phase after 48 hours of ATO treatment, which was not significantly influenced by FAU overexpression. In FAU knockdown cells, ATO treatment did not further increase the amount of cells in G2/M arrest (Supp. Fig. 2B). Even more, in a colony formation assay, arsenic trioxide treatment could not further reduce colony formation after FAU knockdown, whereas cells with FAU overexpression showed a higher sensitivity for this treatment (Fig. 2D). FAU overexpression also sensitizes p53-knockdown cells for ATO treatment (Fig. 2E), suggesting that the increased ATO sensitivity induced by FAU overexpression is not dependent on p53. However, when cells were treated with higher concentrations of this drug, to induce an apoptotic response, no differences were observed between control-, FAU- or FUBI-overexpressing cells, whereas S30 overexpressing cells seemed to be slightly more resistant (Fig. 2F). Interestingly, cells with reduced FAU levels were also more resistant to the high ATO treatment (Fig. 2G).
Effects of FAU expression in osteosarcoma cells

Figure 2. FAU expression enhances sensitivity for ATO treatment, independent of p53.
Stable U2OS cells expressing a control vector or HA-FAU were lentivirally infected with either a control- or two different FAU knockdown vectors. Cells were seeded for WST-1 proliferation assays and colony formation assays 2 days post-infection and treated with 3 µM ATO for 24 hours or mock treated one day after seeding. Proliferation was measured using WST-1 and displayed as relative survival at day 3 compared to the corresponding mock-treated cells (A). Growth curves of mock- or ATO (3 µM 24 hours) treated control and HA-FAU cells are depicted in (B), growth curves of mock- or ATO (3 µM 24 hours) treated control cells or cells that have decreased FAU levels due to expression of one of two different FAU knockdown constructs are depicted in (C). Colony formation of ATO (3 µM 24 hours) treated control- or FAU-knockdown cells is displayed as relative colony formation.
Chapter 7

compared to the corresponding mock treated cells (D). Stable U2OS cell lines expressing either a control vector or an expression vector encoding HA-FAU or short-hairpin RNA’s against p53, or both, were seeded for WST-1 proliferation assays. One day after seeding cells were treated with 3 µM ATO for 24 hours or mock treated. Relative survival of ATO treated cells at day 3 after treatment is depicted in (E). U2OS cells were lentivirally infected with a control vector or an expression vector encoding HA-FUBI, HA-S30 or HA-FAU or either a control-knockdown vector or two different FAU knockdown vectors, and seeded for WST-1 proliferation assays four days post-infection. One day after seeding the cells were treated with the indicated concentrations of ATO for 24 hours or mock-treated. Relative survival of control-, HA-FUBI-, HA-S30 and HA-FAU cells at day 2 after treatment is depicted in (F). Relative survival of control- and FAU-knockdown at day 2 after treatment is depicted in (G).

FAU increases ATO sensitivity of U2OS cells, independently from the ERK1/2 pathway.

Since it has been reported that inhibition of the MEK/ERK1/2 pathway increases ATO sensitivity, we treated control- and FAU-overexpressing cells with arsenic trioxide in the presence or absence of a MEK inhibitor, U0126. Both control and FAU overexpressing cells showed increased ATO sensitivity when co-treated with the MEK inhibitor (Fig. 3A). Indeed, we find that ATO treatment induces phosphorylation of ERK1/2 in both control and HA-FAU overexpressing cells (Fig. 3B). ERK1/2 activation actually functions as a survival signal, thereby attenuating the effect of ATO. Addition of MEK inhibitors U0126 and PD98059 reduced both basal (Fig. 3C, lower panel) and ATO-induced ERK1/2 phosphorylation (Fig. 3C, upper panel). Interestingly, we observed an increase of ERK1/2 phosphorylation upon FAU knockdown, which could still slightly be enhanced by ATO treatment and which was found sensitive to co-treatment with MEK inhibitors (Fig. 3C). A combination treatment with ATO and MEK inhibitors slightly reduced the survival of the FAU knockdown cells, whereas single ATO treatment had no effect (Fig. 3D). Interestingly, treatment with each MEK inhibitor alone seemed to enhance the growth of the FAU knockdown cells somewhat, whereas no effect was observed in the control cells, suggesting that the enhanced ERK1/2 activation in these knockdown cells might have an anti-proliferative function, as has been reported before. Altogether, these results suggest that FAU expression modulates the sensitivity of cells for ATO, independent of ERK1/2 activation. However, the activation of ERK1/2 seems to attenuate the growth inhibiting effect of ATO treatment, independent of the FAU levels. Furthermore, the FAU knockdown experiments suggest that endogenous levels of FAU inhibit basal phosphorylation of ERK1/2, as was published before, although we did not observe decreased phospho-ERK levels in FAU overexpressing cells, suggesting that endogenous levels are sufficient for this function of FAU (Fig. 3B).
Knockdown of FAU and ATO treatment induces gadd45-alpha mRNA expression.

Upon FAU knockdown and also upon ATO treatment we observed an increase of p-ERK1/2, as well as a marked G2/M arrest (Fig. 3C and Supp. Fig. 2B). A candidate protein involved in the biological response to ATO treatment and FAU manipulation is GADD45-alpha. GADD45-alpha is a checkpoint protein, arresting cells at G2/M phase by inhibiting the activity of cyclinB/p34cdc2 complex in response to stress. It has been reported that the transcription of the gadd45-alpha gene can be regulated independently from p53 by the MAPK pathways. Furthermore, it was shown that both JNK1 and ERK1/2 activation could enhance the DNA damage induced GADD45-alpha expression, by interacting with transcription factors such as Oct-1 and NF-YA that bind to OCT-1 and CAAT motifs in the gadd45-alpha promoter. Indeed we found increased gadd45-alpha mRNA expression in cells with reduced FAU levels (Fig. 4A), which might, at least partly, explain the G2 arrest seen upon FAU knockdown. This increase could partially be rescued by FAU overexpression, although it did not affect basal gadd45-alpha expression. ATO treatment also induced gadd45-alpha levels, p53-independently. ATO did not further increase gadd45-alpha in FAU knockdown cells, which already have increased levels (Fig. 4B), which might suggest that ATO and FAU regulate gadd45-alpha via the same pathway. FAU overexpression did not affect the ATO-induced expression of gadd45-alpha (Fig. 4C), indicating that the increased sensitivity of FAU overexpressing cells is not caused by reduced gadd45-alpha induction. Even though these data correlate well with the regulation of phospho-ERK shown in Figure 3, it seems unlikely that the phosphorylation of ERK1/2 is the primary trigger for the induction of gadd45-alpha. On the contrary, the use of MEK inhibitors together with ATO even further enhanced the expression of gadd45-alpha and slightly stimulated the growth inhibitory effect of ATO treatment (Fig. 4C and Fig. 3A). Together, these results suggest that both ATO treatment and reduced FAU expression lead to p53-independent induction of gadd45-alpha, resulting in G2/M arrest, possibly via activation of MAPK pathways, although the observed ERK1/2 activation appears not to be required.

It has also been reported that levels of active, phosphorylated-Akt are transiently upregulated upon ATO treatment but decrease at later time-points. Prevention of the transient activation of Akt strongly increased the apoptotic response. Importantly, Akt is inactivated upon ATO treatment at later time points through induction of c-Cbl proteins. This inactivation of Akt was found to be important for the apoptotic response upon ATO treatment. Moreover, in another study it was found that the inhibition of Akt activation enhanced the ERK1/2 phosphorylation after certain types of stress. Therefore, we analyzed whether FAU overexpression would affect the Akt activation by ATO treatment,
by investigation of phospho-Akt levels and the expression of genes whose expression can be regulated via Akt activation. In both control and FAU cell lines we find Akt inactivated at later time points upon ATO treatment (Fig. 4D).

Figure 3. FAU increases ATO sensitivity independent from the ERK1/2 pathway.
Stable U2OS cells expressing either a control vector or a HA-FAU expression vector were seeded for a WST-1 proliferation assay, 24 hours after seeding cells were treated with 3 µM ATO or 3µM ATO and 10 µM U0126 for 8 hours or mock treated. Relative survival compared to mock-treated corresponding cells 48 hours after start of treatment is displayed in (A). Stable control-vector or HA-FAU expressing U2OS cells were treated with 3 or 5 µM ATO for 24 hours or mock treated, after which protein extracts were isolated. Protein extracts were analyzed with immunoblotting using the indicated antibodies. USP7 expression was used as a loading control (B). U2OS cells were lentivirally infected with either a control- or two different FAU knockdown vectors. At day 2 post-infection cells were treated with 3 µM ATO, 10 µM U0126, 15 µM PD98059 or a combination of ATO and one of both inhibitors for 24 hours, or mock treated. Protein extracts were isolated and analyzed with immunoblotting using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies (C). Relative survival compared to mock-treated corresponding cells 96 hours after start of treatment is displayed in (D).
Effects of FAU expression in osteosarcoma cells

Figure 4. Knockdown of FAU and ATO treatment induces gadd45-alpha mRNA expression.

Stable U2OS cells expressing a control vector or HA-FAU were lentivirally infected with either a control- or two different FAU knockdown vectors. RNA extracts were isolated at day 6 post-infection and GADD45-alpha mRNA levels were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (A). Stable U2OS cells expressing a control- or p53-knockdown vector were lentivirally infected with either a control- or two different FAU knockdown vectors. At day 4 post-infection cells were treated with 3 µM ATO for 48 hours or mock treated and RNA extracts were isolated. Expression levels of GADD45-alpha mRNA were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (B). Stable U2OS cells expressing a control vector or HA-FAU were treated with 3 µM ATO, 3 µM ATO together with 10 µM U0126 or mock-treated for 24 hours. Total RNA was isolated and mRNA levels of GADD45-alpha were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (C). Stable U2OS cells expressing a control vector or HA-FAU were treated with 3 µM ATO for the indicated times, after which protein extracts were isolated and analyzed with immunoblotting, using the indicated antibodies. USP7 expression was used as a loading control (D).
FAU overexpression does sensitize U2OS cells for cisplatin and doxorubicin treatment.

To study the effect of FAU expression on the response to other drugs, we treated control or FAU overexpressing U2OS cells with various concentrations of cisplatin and doxorubicin, enabling us to calculate the effective dose for a certain affected fraction (Fig. 5A and Table 1). FAU overexpressing cells were slightly more sensitive for both cisplatin and doxorubicin; differences were the clearest when concentrations were used that affected more than 90% of the cells. Results were confirmed in colony forming assays, when the cells were treated with low concentrations of either cisplatin or doxorubicin for 24 hours. Again, FAU overexpressing cells were relatively more sensitive (Fig. 5B). However, using these concentrations, it seemed that the cells just arrest their growth but did not show an apoptotic reaction, as no floating cells were observed. Indeed, FACS analysis showed that 24 and 48 hours treatment with 1 µM cisplatin resulted in a large increase in number of G2/M cells of both control- and FAU-overexpressing cells (Fig. 5C). Importantly, hardly any increase in sub-G1 fraction was observed, indicating that this low dose of cisplatin does not notably induce apoptosis at this time-point. Some increase of the sub-G1 fraction was observed when the cells were treated with 0.5 µM doxorubicin (Fig. 5C), although this did not correlate with the increased sensitivity of the FAU overexpressing cells (Supp. Fig. 3A).

Table 1. Effective dose of cisplatin and doxorubicin on U2OS control and FAU-overexpressing U2OS cells.

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U2OS cells stably transduced with either a control vector or HA-FAU expression vector were seeded for WST-1 proliferation assays and colony formation assays. Next day the cells were treated with the indicated concentrations cisplatin or doxorubicin for 24 hours or mock-treated. Proliferation was measured at day 3 after treatment and effective doses were calculated using the CompuSyn programme. Fa = affected fraction. For cisplatin, $r = 0.99422$, and for doxorubicin treatment $r = 0.99337$.

Reduction of FAU increases the resistance of U2OS cells to various drugs

Confirming the effects that were observed in cells that overexpressed FAU, cells with reduced FAU levels were more resistant to cisplatin and doxorubicin. U2OS cells with reduced FAU levels were in a short-term growth assay slightly more resistant to cisplatin (1
Effects of FAU expression in osteosarcoma cells

µM) and significantly more resistant to doxorubicin (0.5 µM) (Fig. 5D). Similar effects were observed in long-term colony assays, in which the growth inhibition by the drugs was more severe. Even so, cells with reduced FAU levels the inhibitory effect of the drug treatment on colony formation was less (Fig. 5E). When cells were treated with higher concentrations cisplatin and doxorubicin, the differences between FAU overexpressing cells and control cells were diminished, but FAU knockdown cells remained more resistant (Supp. Fig. 3B and C). Similarly, when the cells were treated with other drugs, such as Actinomycine D, RITA and Nutlin-3, we observed a reduced sensitivity for the FAU knockdown cells (Supp. Fig. 3D).

Discussion

The FAU gene was reported to exhibit tumor suppressing and pro-apoptotic activities, although also oncogenic functions have been designated to this gene.

It has been shown that the regulation of Bcl-G by FAU is required for its apoptotic functions. In several tumor types the expression of both genes was found to be reduced, correlating with tumor progression, worse patient survival and increased drugs resistance. On the other hand, FAU overexpression has been associated with increased arsenic trioxide resistance and transformation in HOS osteosacoma cells. In that study, the S30 domain was responsible for the increase ATO resistance and the FUBI domain for the transforming ability. Furthermore, FUBI-conjugated Bcl-G was shown to inhibit the MAPK pathway, by inhibiting the phosphorylation of ERK1/2.

In this study, we investigated the role of FAU on the proliferation and drugs resistance in osteosarcoma cell lines. We found a clear growth inhibition, marked by a strong G2/M arrest, as a result of FAU knockdown, which could only be fully rescued by the expression of a complete FAU RNA. In a recently published study, FAU knockdown did not lead to reduced cell viability or apoptosis, however, analyses of cell-cycle progression and growth rate were not performed.

An increase in ERK1/2 phosphorylation was observed upon FAU knockdown, which is consistent with findings that FAU is involved in the inhibition of the MAPK pathway. The growth inhibition caused by FAU knockdown might be the result of GADD45-alpha induction, since we observed increased gadd45-alpha mRNA levels upon FAU knockdown, which could partly be rescued by FAU overexpression. This checkpoint protein can be induced by MAPK activation and induces G2/M arrest. Given the observed enhanced ERK1/2 activity in these knockdown cells and some rescue of the growth inhibition by
Figure 5. FAU overexpression sensitizes cells for cisplatin and doxorubicin treatment.
Stable U2OS cells expressing a control vector or HA-FAU were seeded for WST-1 proliferation assays and colony formation assays. At day 1 after seeding, cells were treated with the indicated concentrations cisplatin or doxorubicin for 24 hours or mock-treated. Proliferation was measured at day 3 after treatment and displayed as relative survival compared to mock-treated corresponding cells (A). Colony formation of control- and HA-FAU cells is displayed as relative colony formation compared to the corresponding mock-treated cells (B). FACS analysis was performed with cells that were treated with cisplatin (1 µM), doxorubicin (0.5 µM) or mock-treated for 48 hours. Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1 (C). U2OS cells were lentivirally infected with either a control- or two different FAU knockdown vectors. At day four post-infection cells were seeded for WST-1 proliferation assays and colony formation assays. One day after seeding, cells were treated with the indicated concentrations cisplatin or doxorubicin for 48 hours or mock treated. Proliferation was measured at day 2 after treatment and displayed as relative survival compared to mock-treated corresponding cells (D). Colony formation of control- and HA-FAU cells, treated for 48 hours with the indicated concentrations and drugs, is displayed as relative colony formation compared to the corresponding mock-treated cells (E).
Effects of FAU expression in osteosarcoma cells

MEK inhibitors, the growth inhibition might be a direct consequence of an activated MEK/ERK cascade. Prolonged ERK1/2 activation has been reported to cause cell cycle arrest and even apoptosis in some circumstances. In contrast to the reported pro-apoptotic function of FAU in other cells, overexpression of FAU did not influence growth of U2OS cells, nor did it inhibit the activation of ERK1/2. Probably an increase in FAU levels only is not sufficient to induce apoptosis. To exert a pro-apoptotic effect FUBI needs to be conjugated to Bcl-G, and Bcl-G levels might be limiting. It is more likely that simultaneous overexpression of both Bcl-G with FAU or FUBI is needed to induce apoptosis. The pro-apoptotic function of FAU is more pronounced when cells are treated with drugs that induce an apoptotic response. Upon various drug treatments, Bcl-G levels will also increase, for instance due to p53 activation. Under these conditions, overexpression of FAU will co-operate with the induced Bcl-G levels, which might explain the observed increased drug sensitivity. Indeed, we find that cells with enhanced FAU levels show increased sensitivity to p53 inducing treatments with ATO, cisplatin or doxorubicin. Importantly, cells with decreased FAU levels were found to be more resistant to these drugs, again indicating that FAU levels are affecting the response to various drugs.

Interestingly, in p53 knockdown cells FAU overexpression did have a sensitizing effect comparable to control cells, indicating a p53-independent mechanism. Since Bcl-G is a p53 target gene, it suggests that more mechanisms are involved in the FAU-mediated drug sensitivity than only the p53-dependent induction of Bcl-G alone. It would, therefore, be interesting if FAU overexpression still sensitizes these p53-negative cells for drug treatments when Bcl-G levels are reduced. It has been reported that Bcl-G knockdown decreases FAU- and UV- induced apoptosis. However, in these experiments just knockdown of Bcl-G resulted in increased basal apoptosis, whereas the total amount of apoptotic cells after FAU transfection or UV treatment was not significantly decreased. So, it is very difficult to interpret these results.

Strikingly, we found some similarities between the effects observed upon FAU knockdown and ATO treatment. ATO treatment induced ERK1/2 phosphorylation, which could be prevented by MEK inhibitors, thereby enhancing the growth inhibiting effect of ATO. These results suggest that ERK activation is pro-survival in this cell system. Depending on the cells used, activation of ERK by ATO has been reported to inhibit or to enhance ATO-induced apoptosis. Arsenic trioxide induced gadd45-alpha expression, which has also been reported to be regulated by JNK and negatively, by NF-κB. FAU knockdown cells also showed induced p-ERK1/2 levels and increased gadd45-alpha mRNA expression,
suggesting again that FAU regulates the activation of some players in the MAPK pathways and might play a role in the equilibrium between proliferative and growth inhibiting factors. FAU expression might be required for optimal proliferation under normal conditions, whereas upon certain types of cellular stress FAU expression might stimulate the apoptotic response. In this model, apparently contrasting results reported previously might be explained. The transforming activity of FAU and FUBI in HOS cells \(^2\), can be explained by the proliferative effects of FAU under normal conditions, whereas its tumor suppressing role is activated under stress conditions.

In conclusion, in this study we show that FAU expression is needed for the normal proliferation of osteosarcoma cells, and that FAU levels influence the sensitivity to several types of drugs, including some that are frequently used in the clinic for osteosarcoma treatment.
Effects of FAU expression in osteosarcoma cells

References

Chapter 7

Effects of FAU expression in osteosarcoma cells

Supplemental Figure 1. Overexpression of HA-tagged FAU, FUBI and S30 in U2OS cells.

U2OS cells were lentivirally transduced with either control vector or expression vectors for HA-FUBI, HA-S30 or HA-FAU. Stable cell lines were established by puromycin selection. Stable cell lines were lentivirally infected with a control- or a FAU knockdown vector. Localisation and expression of the exogenous HA-tagged proteins was determined by immunofluorescence with anti-HA antibodies. DAPI staining was used to visualise nuclei (A). Co-localisation of exogenous HA-S30 and the nucleolar protein B23 was visualized in stable control or HA-S30 expressing U2OS cells using immunofluorescence with anti-B23 and anti-S30 antibodies. DAPI staining was used to visualise nuclei and merged images are shown in the most right panel (B). Protein and mRNA extracts of stable control-, HA-FAU, HA-FUBI and HA-S30 expressing U2OS cells were isolated 6 days after lentiviral infection with control- or FAU knockdown vectors. Expression levels of endogenous FAU, total FUBI and total S30 mRNA were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (C). Protein extracts were analyzed with immunoblotting using an anti-HA antibody, USP7 expression was used as loading control (D).
Supplemental Figure 2. FAU overexpression rescues *FAU*-knockdown induced G2/M arrest; *FAU* knockdown cells are resistant to ATO treatment.

Stable U2OS cells expressing a control vector or expression vectors for HA-FAU, HA-FUBI or HA-S30 were lentivirally infected with either a control- or two different *FAU* knockdown vectors. At day 6 post-infection cells were harvested for FACS analysis (A). U2OS cells were lentivirally infected with a control vector, an HA-FAU expression vector or a vector expressing a short hairpin RNA against *FAU*. At day 4 post-infection, cells were treated with 3 µM ATO for 48 hours or mock treated and harvested for FACS analysis (B). Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1.
Supplemental Figure 3. Reduction of FAU levels decreases sensitivity of U2OS cells for various drugs at high concentrations.

U2OS cells stably expressing a control vector or HA-FAU were seeded for WST-1 proliferation assays. Next day, cells were treated with the indicated concentrations cisplatin or doxorubicin for 24 hours or mock treated. Proliferation was measured 48 hrs later and displayed as relative survival compared to mock-treated corresponding cells (A). U2OS cells were lentivirally infected with either a control vector or an expression vector encoding HA-FAU and seeded for WST-1 proliferation assays four days post-infection. One day after seeding, cells were treated with the indicated concentrations of cisplatin or doxorubicin and cell viability was determined 48 hrs later and displayed as relative survival compared to mock-treated corresponding cells (B). U2OS cells were lentivirally infected with either a control-knockdown vector or two different FAU knockdown vectors, and seeded for WST-1 proliferation assays four days post-infection. One day after seeding, cells were treated with the indicated concentrations of the indicated drug. Proliferation of cisplatin or doxorubicin-treated cells (C) and of cells treated with actinomycin D, RITA or Nutlin-3 (D) was measured after 48 hrs of treatment and displayed as relative survival compared to mock-treated corresponding cells.
## Supplemental Information

### Q-RT-PCR Primer Sequences

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Chapter 8

Summary and General Discussion
Chapter 8

The p53 tumour suppressor pathway is one of the best studied pathways in cancer, indicating its importance in this field. Regulation of p53 is critical for normal functioning of any cell or organism. Therefore, the understanding of the function and role of the main regulators of p53, Hdm2 and Hdmx, is a crucial part of these studies. Whereas Hdm2, which was identified as a p53 binding protein 5 years before its homolog Hdmx 1,2, has been extensively studied, (almost 4700 articles), studies featuring Hdmx are still underrepresented (only ~300 articles), although the last few years this number is steadily growing. It has now become clear that Hdmx is also an essential negative regulator of p53, given the non-redundancy in knock-out mice 3-7 and, moreover, the high frequency of Hdmx overexpression in certain types of tumours 8-10, indicating an oncogenic function. In this thesis we have investigated and now discuss the role of Hdmx overexpression in transformation and tumourigenesis in several model systems. The regulation of the Hdmx-p53 interaction is described in various ways; post-translational modifications on the Hdmx protein and alternative splicing of the Hdmx mRNA may all influence the function of Hdmx.

Regulation of Hdmx

Post-translational modifications of the members of the p53 pathway, mediated by several upstream activators, contribute to p53 activation upon DNA damage 11. Phosphorylation of P53, Hdm2 and Hdmx may lead to reduced interaction between p53 and its negative regulators, whereas the Hdm2-mediated degradation of Hdmx is enhanced 12-14, due to reduced USP7 activity towards Hdmx 15. The c-Abl non-receptor tyrosine kinase has been reported to play an important role in the p53 response by neutralizing the inhibitory effects of Hdm2 16,17. We could show that not only Hdm2 is phosphorylated by c-Abl, but that also Hdmx is target of this kinase, with tyrosines 55 and 99 as main phosphorylation sites (This thesis, Chapter 3, 18). Interestingly, these sites are within the p53 binding domain and it was demonstrated that the DNA damage-induced phosphorylation on Y99 inhibits the binding between p53 and Hdmx, thereby providing an additional mechanism for the activation of p53 after DNA damage. Even more, the phosphorylation of Y55 seemed to counteract the effect of Y99 phosphorylation and might be involved in the recovery phase after stress, when reduced activity of p53 is required.

These findings indicate the importance of Hdmx in both the p53 response and the regulation of the recovery after DNA damage.
Splicing of Hdmx into Hdmx-S regulates the amount of full length Hdmx protein.

One of the characteristics of the p53 response is a reduction of Hdmx protein. This is usually the result of induced Hdm2 levels and Hdm2-mediated ubiquitination activity towards Hdmx. However, we show that there might be an additional mechanism to reduce Hdmx levels upon DNA damage, which also functions in certain stages of tumour formation (This thesis, Chapter 2 and 5). The alternative splice variant Hdmx-S encodes the Hdmx-S protein, which consists essentially only of the p53 binding domain plus a few unique C-terminal amino acids and this shorter protein was initially reported to be a more potent p53 inhibitor. We demonstrated that the upregulation of Hdmx-S mRNA expression after DNA damage is accompanied by reduced full length Hdmx protein levels. Furthermore, we found a relatively increased Hdmx-S mRNA expression in osteosarcoma cell lines which express low levels of Hdmx protein, mainly correlating with inactivated p53. Moreover, we have shown that relatively high Hdmx-S mRNA expression in osteosarcoma tumours is correlated with a more aggressive clinical stage of the tumour, characterized by significant higher incidence of metastasis formation and worse disease-related survival, thereby functioning as a prognostic factor. However, we also demonstrated that the endogenous Hdmx-S protein is hardly expressed, as it is not detectable, and therefore, most likely is not functional active. Thus, we proposed that increased Hdmx-S expression is mainly a way to reduce full length Hdmx protein levels. The association between a high ratio Hdmx-S/Hdmx-FL mRNA and mutant or inactivated p53 was confirmed in two independent panels of cell lines. In the NCI60 cell panel, consisting of 60 cell lines derived from different tumour types, high Hdmx-S expression was clearly associated with p53 mutation and Hdm2 overexpression. Similar results were obtained with a panel of 39 breast cancer tumour cell lines, which were characterized recently.

Furthermore, in a set of soft tissue sarcoma patients the increased Hdmx-S/Hdmx-FL ratio turned out to be a better predictor for overall survival than the mutational status of p53 (This thesis, Chapter 5).

These results combined strongly indicate that increased Hdmx-S mRNA expression mainly functions as a way to reduce the functional, full length Hdmx protein. This mechanism is activated upon various p53 activating treatments in normal and tumour cells, whereas it seems to be constitutively active in certain tumour cells. Although reduction of Hdmx as an advantage for tumour development might be counterintuitive, it might be that Hdmx expression may exert growth repressive functions in p53-negative or mutant tumours.

Matijasevic et al. demonstrated in p53-negative MEFs that Mdmx inhibited transformation and tumourigenesis by promoting bipolar mitosis. These findings fit in a model where in later stages of tumourigenesis, tumours with non-functional p53 select for reduced Hdmx.
expression, to reduce its anti-oncogenic activity, thereby enhancing their tumourigenic capabilities.

Oncogenic functions of Hdmx

Although downregulation of Hdmx might enhance the tumourigenicity and especially the aggressiveness of p53-negative tumour cells, amplification and overexpression of Hdmx is frequently found in several tumour types 8-10 or tumour cell lines 26. The Hdmx overexpression usually correlates with wild-type p53 status, suggesting an oncogenic role for Hdmx. The oncogenic function of Mdmx in mouse cells was demonstrated by Danovi et al. (2004) 8; in human cells this was not clearly proven yet. We have shown that the overexpression of Hdmx could replace the loss of p53 during the transformation process of human fibroblasts and embryonic retinoblasts (This thesis, Chapter 4). Several growth aspects such as proliferation rate, anchorage-independent growth and in vivo tumour growth were induced by exogenous Hdmx overexpression, comparable to loss of p53. Furthermore, we demonstrated in the same study that high levels of Hdmx can affect the response to the p53 activator Nutlin-3.

Confirming these results, we also have shown that Hdmx knockdown in osteosarcoma cells is growth repressive and enhanced the effect of Nutlin-3, cisplatin and doxorubicin. These effects were partly p53-dependent, but growth inhibition upon Hdmx-knockdown was also observed in p53 mutant cells (This thesis, Chapter 5). Altogether, these results indicate that Hdmx overexpression can indeed exert an oncogenic function and may cooperate with other factors in the neoplastic transformation of human cells. We have demonstrated that Hdmx overexpression enhanced the resistance to chemotherapeutic drugs, by inhibiting the p53 response, whereas reducing Hdmx levels increased the sensitivity for p53 reactivating drugs. Therefore, the development of Hdmx inhibitors 27,28 in combination with conventional drugs may provide a new way of treatment for a specific set of tumour types. Especially tumours with wild-type p53 and high Hdmx, which are relatively resistant to Hdm2 inhibitors 29, may be treated more efficiently with these new type of drugs. However, considering our findings, it might be that highly transformed tumours with inactivated p53 will have an opposite response to Hdmx inhibitors, since we found that in these tumours a selection for down regulation of Hdmx may occur (see above).

SUMOylation and ubiquitination in the p53 pathway

Ubiquitin-like modifications play an important role in the regulation of cell growth, proliferation and the responses to stress, such as growth arrest or apoptosis. Especially in the p53 pathway, ubiquitin functions as a regulator of activity and proteasomal degradation...
Summary and General Discussion

The regulation of p53 levels is for a large part controlled at the protein level, via proteasomal degradation. The amount of Hdm2 and Hdmx is also partly regulated at the protein level, although transcription from the p53-responsive Hdm2 promoter is greatly induced upon p53 activation. The net result of Hdm2-mediated ubiquitination and de-ubiquitination by DUB’s like USP7 determines the rate of degradation of p53, Hdm2 and Hdmx and, thereby, the fate of the cell. The ubiquitination and de-ubiquitination is influenced in various ways by post-translational modifications, like phosphorylation, as mentioned above, but also other modifications, such as SUMOylation. The conjugation of SUMO may lead to several possible outcomes: SUMOylation of components of the ubiquitination system may enhance or reduce the ubiquitination process, SUMOylation of a target protein may compete with ubiquitination or might enhance it. A SUMOylated protein may serve as a target for SUMO Targeted Ubiquitin Ligases, which specifically recognize and ubiquitinate (poly)-SUMOylated proteins. The finding of a huge increase of high molecular weight species in the SUMOylated fraction of p53, Hdm2 and Hdmx after proteasomal inhibition, might suggest that SUMOylation plays a role in the ubiquitination of these proteins (This thesis, Chapter 4). We found that hypoxia and MAPK signalling can influence the SUMOylation status of p53 and its regulators, thereby indirectly also affecting their ubiquitination.

The increased SUMO-dependent degradation of Hdm2 and Hdmx and decreased SUMO-dependent ubiquitination of p53 observed after DNA damage (this thesis, Chapter 4) confirms previous findings, as it was suggested that SUMOylation of p53 inhibits its activity, whereas Hdm2 SUMOylation stimulates auto-ubiquitination. Although we could not detect any differences in SUMOylation of Hdmx after DNA damage, the SUMOylation of Hdm2 might indirectly regulate Hdmx levels. To elucidate the function of Hdmx-SUMOylation, extensive studies are required, comparing the biological responses of SUMO-deficient Hdmx mutants and wild-type Hdmx expressing cells upon various treatments.

Furthermore, we investigated the role of the human STUbL RNF4 in the SUMO-dependent ubiquitination of p53, Hdm2 and Hdmx. It has been reported that RNF4 specifically ubiquitinates poly-SUMOylated PML after arsenic trioxide treatment, leading to PML protein degradation. Interestingly, while wild-type p53 is activated and stabilized by arsenic trioxide treatment, it has been reported that mutant p53 is degraded by this treatment. Mutant p53 was reported to interact with PML, leading to enhanced activity. These results suggest that a role of RNF4 in the regulation of p53 is well possible, although we could not find indications for a direct role of RNF4 in the SUMO-dependent ubiquitination of p53, Hdm2 and Hdmx. Nevertheless, RNF4 still might be involved in the regulation of the p53 pathway. Slight changes in of p53, Hdm2 and Hdmx
protein levels were observed upon RNF4 knockdown or overexpression, indicating an indirect role of RNF4 on the regulation of p53. Especially the RING-dependent stabilizing effect of RNF4 on Hdm2 might be an interesting subject for further study. Strikingly, we observed a downregulation of RNF4 when cells were treated with various drugs, which was correlated to the induction of apoptosis and the sensitivity to the treatment. Moreover, we found that increased RNF4 expression resulted in a reduced apoptotic response after treatment with the p53 activating agent RITA. The role of RNF4 in the DNA damage response is not fully clear yet. It has been reported to be required for DNA damage repair\textsuperscript{47}, and our results suggest that RNF4 might also be involved in the fine-tuning of the p53 response after stress or DNA damage. These functions might implicate RNF4 as an oncogene, since overexpression might reduce the tumour-suppressing function of p53. Enhanced RNF4 expression has been associated with tumour formation in mouse models and found to be enhanced in breast tumours\textsuperscript{48}. The expression level of RNF4 might serve as a prognostic factor for therapy response and drug targeting of RNF4 in tumours could enhance the effect of chemotherapeutic treatments.

**Role of FAU in growth and drugs resistance**

Whereas ubiquitin- and SUMO-chain conjugation on target proteins may lead to altered localisation, function or degradation\textsuperscript{49}, the ubiquitin-like FUBI is not likely to be able to form poly-chains, since it lacks most of the internal lysines, which are, in ubiquitin, required for chain formation. The only conserved lysine in FUBI, K27, might be used for poly-linkages\textsuperscript{50}, but it is unlikely that this type of chain would lead to proteasomal degradation. The *FAU* gene, which encodes FUBI and the ribosomal protein S30, has been associated with apoptosis in several studies\textsuperscript{51-55}. The conjugation of FUBI to the p53 target Bcl-G was reported to contribute to the pro-apoptotic function of FAU\textsuperscript{52-55}. Furthermore, *FAU* mRNA expression is down-regulated in a number of tumour types\textsuperscript{53,54,56,57} and overexpression of FAU enhances the apoptotic effect of carboplatin and UV-C treatment\textsuperscript{53,54,57}. We report in this thesis, Chapter 5, that *FAU* knockdown is growth-inhibiting, indicating a function for FAU in cell growth. In contrast to what has been reported before\textsuperscript{51,55}, we did not observe increased basal apoptosis after FAU overexpression. Since conjugation of FUBI to Bcl-G has been demonstrated to be required for the FAU-induced enhanced apoptosis, the basal levels of Bcl-G might be limiting for the pro-apoptotic effect in these cells. Furthermore, these effects might be cell-type specific. Importantly, we demonstrated that FAU overexpression enhances the growth repressive effect of arsenic trioxide, cisplatin and doxorubicin. Consistently, cells which have reduced FAU levels are more resistant to these treatments. These results are confirming previous reports\textsuperscript{53,54,57} which are indicating FAU as a pro-apoptotic factor. Although it is the FUBI part that
interacts with Bcl-G\textsuperscript{52}, our results suggest that the complete \textit{FAU} sequence is required for the apoptosis enhancing effect. Moreover, in the initial functional expression cloning studies\textsuperscript{51}, which identified the \textit{FAU} anti-sequence to be anti-apoptotic, the whole anti-\textit{FAU} sequence was found, indicating again that the complete sequence is needed. Interestingly, in cells that have reduced p53 mRNA levels, FAU overexpression still sensitizes for arsenic trioxide growth inhibition, suggesting that p53 activation is not essential for the FUBI-mediated apoptotic response.

Further investigations concerning the role of FAU in the response to drugs are required, since it might be a potential new target for anti-tumour therapy.
References

Summary and General Discussion

Nederlandse Samenvatting
Alle levende wezens zijn opgebouwd uit cellen en elke cel bevat de volledige genetische informatie van het organisme, het genoom, opgeslagen in het DNA. De coderende delen van het DNA worden genen genoemd, welke elk voor één of meerdere eiwitten coderen. De genen bevatten specifieke informatie over hoe een eiwit is opgebouwd, namelijk de precieze volgorde van de aminozuren, de bouwstenen van eiwitten. Om een eiwit te maken, wordt eerst het desbetreffende stukje DNA overgeschreven in RNA; dit proces wordt ook wel transcriptie genoemd. Dit stukje RNA fungeert daarna als een bouwtekening om een eiwit te maken, in een proces dat translatie wordt genoemd. Aangezien de eiwitten cruciale rollen spelen in bijna elk proces in de cel, is het erg belangrijk dat deze informatie blijft bestaan en wordt doorgegeven. Daarom wordt bij elke celdeling het DNA verdubbeld en gelijk verdeeld over de dochtercellen. Tijdens dit proces, replicatie genoemd, wordt er voortdurend gecontroleerd op fouten of beschadigingen in het DNA, omdat veranderingen in het DNA, mutaties genoemd, schadelijk kunnen zijn voor de cel en daardoor mogelijk voor het hele organisme. Het DNA verdubbelingsproces wordt gestaakt als er een fout wordt ontdekt, en pas hervat als de schade is gerepareerd. Als de schade zo groot is dat betrouwbaar herstel niet meer mogelijk is wordt er een geprogrammeerde celdood, apoptose genoemd, opgestart. Als deze controlemechanismen niet meer werken kunnen beschadigde cellen ongehinderd verder delen en daardoor neemt de kans op het ontstaan van kanker aanzienlijk toe. Het is dus voor het organisme beter dat er één cel dood gaat, dan dat het gehele organisme in gevaar komt door ongeremde celgroei.

groot verschil is dat Hdm2 voor de afbraak van p53 zorgt, terwijl Hdmx voornamelijk de activiteit van p53 remt. Hdmx kan deze functie uitvoeren door te binden aan het p53 eiwit en daarmee te verhinderen dat p53 de transcriptie van zijn target genen kan activeren. Veel is al bekend is over functies en regulatie van Hdm2, maar er is nog relatief weinig onderzoek gedaan over Hdmx.

In hoofdstuk 2 van dit proefschrift wordt de huidige kennis over Hdmx samengevat, waarbij vooral de verschillende manieren van Hdmx regulatie aan bod komen.

In hoofdstuk 3 wordt beschreven hoe Hdmx gemonitoced wordt na DNA schade door het signaal eiwit c-Abl. Dit eiwit wordt actief na DNA schade en plaatst fosfaatgroepen op een bepaald aminozuur van eiwitten, waaronder Hdm2 en Hdmx. Het plaatsen van deze fosfaatgroepen, fosforlyering, beïnvloedt de functie van deze eiwitten. We laten zien dat Hdmx op 2 aminozuren, tyrosines, wordt gefosforlyeerd door c-Abl, waarvan de fosforlyering op tyrosine 99 zorgt voor vermindere binding tussen Hdmx en p53. Dit leidt tot verminderde remming van p53 activiteit door Hdmx en deze modificatie draagt dus bij aan de activatie van p53 na DNA schade.

In hoofdstuk 4 en 5 wordt de oncogene functie van Hdmx beschreven. Met andere woorden, welke rol speelt Hdmx in het ontstaan van kanker? Voor veel verschillende tumor soorten is beschreven dat het Hdmx gen verhoogd tot expressie komt, wat meestal correleert met het behoud van functioneel p53. Deze waarnemingen suggereren dat een verhoogde hoeveelheid van het Hdmx eiwit de tumor suppressor functie van p53 kan onderdrukken. Met gebruik van muizencellen heeft men al laten zien dat verhoogde expressie van Hdmx, dat in muizen Mdmx genoemd wordt, kan leiden tot transformatie van normale cellen naar tumorogene cellen. In menselijke cellen was nog geen direct bewijs geleverd voor deze oncogene activiteit van Hdmx. In hoofdstuk 4 laten we zien, in een studie met gebruik van twee soorten normale menselijke cellen, dat verhoogde expressie van Hdmx bijdraagt aan de transformatie van deze cellen, vergelijkbaar met het effect dat het verlies van p53 veroorzaakt. In een vergelijkende studie tonen we dat verhoogde expressie van Hdmx, net als het verlies van p53, verschillende groei-aspecten van menselijke cellen versnelt en ook de activatie van p53 na behandeling met p53 activerende stoffen kan voorkomen.

In hoofdstuk 5 wordt de rol van Hdmx in osteosarcoma beschreven, een vorm van tumoren die vooral bij jonge mensen voorkomt. Omdat slechts in ongeveer 20% van deze tumoren een defect in het p53 eiwit wordt aangetroffen, en in een vergelijkbaar deel een verhoogde hoeveelheid Hdm2 wordt gevonden, ligt een rol voor verhoogde Hdmx expressie in het ontstaan van deze tumoren voor de hand. Wij hebben de hoeveelheid Hdmx
onderzocht in 51 osteosarcoma tumormonsters, en in 22 osteosarcoma cellijnen. Hoewel er slechts in enkele gevallen een verhoogde expressie van Hdmx werd gevonden, konden we laten zien dat Hdmx nodig is voor de groei van cellen met functioneel p53 en ook van p53-mutant cellen, duidend op een deels p53-onafhankelijke functie van Hdmx. Verder laten we zien dat er in een hoog percentage van de osteosarcoma tumoren en cellijnen verhoogde expressie is van een alternatief Hdmx mRNA, Hdmx-S. Als een gen wordt overgeschreven naar RNA, worden alleen de relevante delen van dat gen (exonen) in de RNA codering gezet, door stukken die niet voor RNA coderen eruit te laten; dit proces wordt RNA-splicing genoemd. Als een RNA op een alternatieve manier gespliceerd wordt, bijvoorbeeld door een coderend stukje (exon) over te slaan, ontstaat er na vertaling van dat mRNA vaak een (gedeeltelijk) ander eiwit. Het alternatieve Hdmx-S mRNA mist het coderende gedeelte van exon 6, resulterend in een verkort Hdmx eiwit, dat slechts het p53 bindingsdomein van het oorspronkelijke Hdmx bevat. Volgens sommige eerdere publicaties is dit korte Hdmx eiwit een betere p53 remmer en betrokken bij de tumorvorming. Wij laten zien dat het Hdmx-S RNA wel in vele tumoren en tumor cellijnen tot expressie komt, maar dat het Hdmx-S eiwit zeer onstabiel is of zeer inefficiënt tot expressie wordt gebracht en zeer waarschijnlijk geen belangrijke functie vervult. Dat deze RNA variant toch verhoogd tot expressie komt in veel tumoren, is mogelijk een manier om de hoeveelheid functioneel volledig Hdmx eiwit omlaag te brengen. We laten zien dat een verhoogde Hdmx-S expressie samenhangt met verlaagde hoeveelheid Hdmx en meestal met inactief p53. Bovendien hebben kanker patiënten met een hoge ratio Hdmx-S / Hdmx in de tumor een kortere overleving en lopen een significant hoger risico op het krijgen van uitzaaingen. Op grond van deze resultaten poneren wij een model dat stelt dat het volledige Hdmx eiwit bij het ontstaan van de tumor een rol speelt door p53 activiteit te remmen. Maar in tumoren die zich in latere, meer agressieve stadia bevinden en waarin p53 gemuteerd of op andere wijze geïnactiveerd is, heeft Hdmx een groeiremmende functie en wordt door middel van alternatieve splicing de hoeveelheid functioneel Hdmx verlaagd. Door meerdere onafhankelijke panels van verschillende tumorcellijnen en patiënt data te analyseren hebben we dit model kunnen valideren.

In hoofdstuk 6 worden andere eiwitmodificaties beschreven, namelijk ubiquitinering en SUMOylering. Dit betekent het koppelen van ubiquitine en SUMO aan andere eiwitten. Ubiquitine en SUMO zijn vergelijkbare kleine eiwitjes die dienen als signaalmoleculen. Het koppelen van deze kleine eiwitten aan een ander eiwit kan bijvoorbeeld de activiteit, de plaats in de cel en de stabiliteit van dat andere eiwit beïnvloeden. Het vormen van een ketting van ubiquitines aan een eiwit is een signaal om dat eiwit af te breken. We laten zien dat zowel p53 als Hdm2 en Hdmx geSUMOyleerd worden en dat specifiek deze
geSUMOyleerde eiwitten dan geubiquitineerd en afgebroken worden. De rol van RNF4, een SUMO-specifieke ubiquitine ligase, dat wil zeggen een eiwit dat ubiquitine aan andere eiwitten koppelt als daar al een SUMO aan zit, is onderzocht. Hoewel we geen directe regulatie van de SUMO-afhankelijke ubiquitinering door RNF4 hebben kunnen vinden, lijkt RNF4 wel een rol in de regulatie van p53 en zijn regulators te hebben, evenals in de DNA schade response.

Een minder bekend familielid van de ubiquitine-achtige eiwitten, FUBI, wordt beschreven in hoofdstuk 7. Over de functie van FUBI, dat als een fusie-eiwit samen met het (ribosomale) eiwit S30 van het FAU mRNA wordt geproduceerd, is nog niet veel bekend. Expressie van het gehele FAU gen zou de apoptotische reactie versterken na behandeling met verschillende chemotherapeutica. Dat zou kunnen verklaren waarom in sommige tumortypes de expressie van FAU verlaagd is. Het FUBI deel van het FAU eiwit zou gekoppeld worden aan het pro-apoptotische eiwit Bcl-G en deze binding is nodig voor een sterke apoptotische respons. In deze studie tonen wij aan dat de expressie van het gehele FAU gen nodig is voor normale groei van osteosarcoma cellen, terwijl overexpressie van FAU de cellen gevoeliger maakt voor verschillende chemotherapeutica, zoals cisplatina, doxorubicine en arsenicum trioxide, welke onder andere worden gebruikt voor de behandeling van osteosarcoma in de kliniek.

Het werk in dit proefschrift biedt meer inzicht in de rol van Hdmx in het ontstaan van tumoren (kanker) en kan hierdoor een bijdrage leveren aan het ontwikkelen van nieuwe behandelmethode voor sommige tumoren. Bovendien is meer inzicht verkregen in de regulatie van hoeveelheid actief Hdmx, zowel op eiwit niveau door middel van modificaties, als op RNA niveau, door de alternatieve splicing van het Hdmx RNA. Daarbij geven we in dit werk meer inzicht in de rol van het FAU gen met betrekking tot normale celgroei en resistentie tegen chemotherapeutica, hoewel meer onderzoek nodig is om een mechanismistische verklaring te vinden voor de werking van de bijbehorende eiwit producten.
Curriculum Vitae

Kristiaan Jan Lenos was born on the 14th of February 1983 in Delft, the Netherlands. In the period from September 1995 until July 2001 he followed his pre-university education at College Hageveld Atheneum in Heemstede. In September 2001 he started with the study Life Sciences & Technology at the Technical University of Delft and the University of Leiden. During this study, a bachelor internship was done at the Molecular Cell Biology department of the University of Leiden, in the lab of Dr. C. Backendorf, and an industrial internship at the Target Discovery Department of Solvay Pharmaceuticals. The Master degree was obtained in 2006 after a research internship at the lab of Dr. AG Jochemsen at the Molecular Cell Biology department of the Leiden University Medical Center. This internship resulted in a PhD position in the same group, which was initiated in November 2006 under supervision of Dr. AG Jochemsen. From September 2011 he started working as a post-doc in the lab of Prof. dr. Yvette van Kooyk under supervision of Dr. ing. Sandra van Vliet at the Department of Molecular Cell Biology and Immunology of the Vrije Universiteit Medical Center.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
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<td>ATO</td>
<td>Arsenic Trioxide</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal Regulated Kinase</td>
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<td>CAM</td>
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<td>COBRA-FISH</td>
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<td>FAU</td>
<td>FBR-MuSV-Associated Ubiquitously expressed</td>
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<td>FBR-MuSV</td>
<td>Finkel-Biskis-Reilly murine sarcoma virus</td>
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<td>GADD45-alpha</td>
<td>Growth Arrest and DNA Damage-inducible protein 45a</td>
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