Functions and regulation of Hdmx and post-translational modifications in drug sensitivity and cancer.

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Chapter 1

General Introduction

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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).

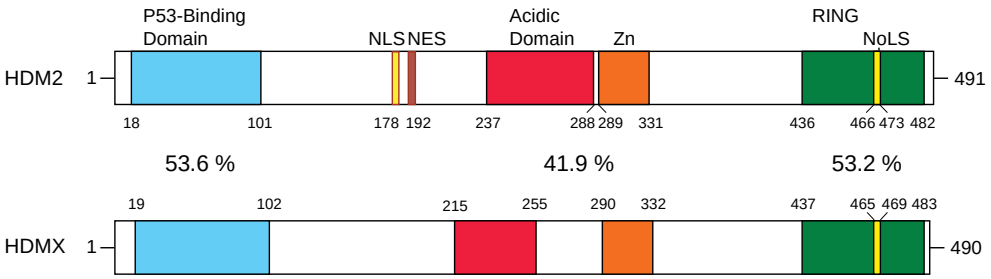

Figure 1. Protein structure of Hdm2 and Hdmx

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 [17,18]. Hdm2 is an E3 ubiquitin ligase for p53, targeting p53 for ubiquitination and proteasomal degradation [19,20], which is dependent on the RING and acidic domains [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 [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 [26-28].

Other ubiquitin E3 ligases for p53 have been described [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 [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 [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 [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 [34,36-38].

Several alternative splice variants of *Hdm2* have been described [39] and up to now six alternative spliced forms of *Hdmx* have been detected [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 [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 [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 [47] and a subset of gliomas showed amplification of the *Hdmx* gene [43]. In a large number of

wild-type p53 tumour cell lines upregulated or aberrant Hdmx protein expression was found [48]. 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 [49]. Moreover, around 90% of Ewing Sarcomas show wild-type p53 and a high number of these tumours show Hdmx overexpression [50].

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 [47]. Recently, two transgenic mouse models have been described that widely overexpress the *Mdmx* gene [51,52]. 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 [53-55] and *Hdm2* was found to be amplified in only 10-35% of osteosarcoma [53-56], 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 [57], have already been used in many studies and are being tested in clinical trials. More recently, also specific inhibitors of Hdmx have been developed [58,59]. 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 [60]. 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 [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 [64-66]. Hdm2 and Hdmx are also SUMOylated on two or more lysines, which have been implicated in the function of Hdm2 [67-70]. Interestingly, the conjugation of SUMO and ubiquitin cross-talk in several ways [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 [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 [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 [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 [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 [83,84]. Phosphorylation of S46 is associated with the induction of apoptosis [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 [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 [86]. Phosphorylation of Hdmx C-terminal serines S342, S367 and S403 promotes its degradation by Hdm2 [87-89]. Similarly, the phosphorylation of Hdm2-S395 by ATM induces auto-ubiquitination of Hdm2 [90]. Furthermore, c-Abl-mediated phosphorylation of Hdm2 on the adjacent Y394 inhibits the nuclear export and degradation of p53 [91-93]. Additionally, phosphorylation of S17 on Hdm2 by DNA-PK has been reported to disrupt the Hdm2-p53 binding [94] and we have shown that tyrosine-99 phosphorylation of Hdmx by c-Abl exerts a similar function (this thesis, Chapter 3, [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 [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 [78,98,99].

The *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 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 *FAU* gene was originally identified as a pro-apoptotic gene [100] and its

downregulation is associated with tumourigenicity and drug resistance [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 [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 [107]. In this thesis, Chapter 7, we investigated the function of the *FAU* gene in osteosarcoma and found, in contrast to what was published before, that *FAU* expression is essential for normal proliferation. Furthermore, we demonstrated that *FAU* expression affects the sensitivity to several drugs.

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