The handle http://hdl.handle.net/1887/18929 holds various files of this Leiden University dissertation.

**Author:** Lange, Job de

**Title:** A sight for sore eyes : assessing oncogenic functions of Hdmx and reactivation of p53 as a potential cancer treatment

**Date:** 2012-05-09
Chapter 3

High levels of Hdmx promote cell growth in a subset of uveal melanomas

J. de Lange\textsuperscript{1}, A.F.A.S. Teunisse\textsuperscript{1}, M. Verlaan-de Vries\textsuperscript{1}, K. Lodder\textsuperscript{1}, S. Lam\textsuperscript{1}, G.P.M. Luyten\textsuperscript{2}, M.J. Jager\textsuperscript{2}, A.G. Jochemsen\textsuperscript{1}

\textsuperscript{1} Department of Molecular Cell Biology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands
\textsuperscript{2} Department of Ophtalmology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands
Chapter 3

Abstract

The p53 tumor suppressor pathway is inactivated in cancer either via direct mutation or via deregulation of upstream regulators or downstream effectors. P53 mutations are rare in uveal melanoma. Here we investigated the role of the p53 inhibitor Hdmx in uveal melanoma. We found Hdmx over-expression in a subset of uveal melanoma cell lines and fresh-frozen tumor samples. Hdmx depletion resulted in cell-line dependent growth inhibition, apparently correlating with differential Hdm2 levels. Surprisingly, p53 knockdown hardly rescued cell cycle arrest and apoptosis induction upon Hdmx knockdown when using three different shRNA constructs, whereas it effectively prevented growth suppression induced by the potent p53 activator Nutlin-3. These findings suggest a novel, growth-promoting function of Hdmx that does not rely on its ability to inhibit p53. We provide evidence for a contribution of p27 protein induction to the observed p53-independent G1 arrest in response to Hdmx knockdown. In conclusion, our study establishes the importance of Hdmx as an oncogene in a subset of uveal melanomas and widens the spectrum of its function beyond p53 inhibition.
High levels of Hdmx promote cell growth in a subset of uveal melanomas

Introduction

After skin, primary melanoma most commonly affects the eye [1]. Uveal melanoma arises in the uveal tract, which comprises the iris, ciliary body, and the choroid. Current treatments mostly involve plaque radiotherapy (brachytherapy), proton beam irradiation or enucleation [2]. However, these local treatments do not prevent distant metastases. Up to 50% of patients with uveal melanoma develop metastases after the initial diagnosis and treatment, most frequently in the liver. Prognosis is poor when the tumor has metastasized; metastases are only sporadically curable and median survival is about 10 - 18 months [3;4]. Therefore, a better understanding of the molecular mechanisms underlying uveal melanomagenesis is needed to develop more efficient treatment modalities.

The molecular pathogenesis of uveal melanoma is different from that in cutaneous melanoma. For example, mutations of NRAS, BRAF and CDKN2A (the gene encoding p16^{INK4A} and p14^{ARF}) are frequently observed in cutaneous melanoma, but not in uveal melanoma [5-7]. Uveal melanomas have been reported to show frequent loss of chromosome 3, correlating with poor prognosis [8]; over-expression of Cyclin D1 [9;10] and inactivating mutation of BAP1 [11], both associated with metastasis; activating mutations of GNAQ and GNA11 [12;13] and promoter methylation of the tumor suppressors p16^{INK4A} [14] and RassF1A [15]. Interestingly, mutations of p53 are uncommon both in cutaneous melanoma [16] and in uveal melanoma [17-19]. DNA damage induces p53 stabilization in uveal melanoma cell lines, although downstream functional defects may be common [20].

Functional inactivation of the p53 tumor suppressor pathway is believed to be involved in virtually all human cancers [21]. Direct gene mutation is found in about 50% of tumors [22;23], whereas those retaining wild type p53 contain other genetic changes preventing p53’s tumor suppressor function [24]. P53 maintains genomic integrity following a variety of stress signals by orchestrating the cellular responses, including cell cycle arrest, DNA repair, senescence and apoptosis [25]. Controlled p53 activation requires tight regulation of the main p53 inhibitors, Hdm2 and Hdmx [26]. Hdm2 ubiquitinates p53 to target it for degradation [27], whereas Hdmx functions mostly by inhibiting p53 activity through interaction with its transcription activation domain [28;29]. Furthermore, Hdmx and Hdm2 dimerize via their RING finger domains [30], which promotes Hdm2’s E3 ligase activity towards p53 [31;32].
About 5-10% of all human tumors show Hdm2 overexpression [33]. In addition, increased Hdmx mRNA levels in 20% of common tumor types [34] and Hdmx gene amplification and overexpression in high percentage of retinoblastomas [35] and in a subset gliomas [36] indicate an oncogene function for Hdmx. Aberrant Hdmx expression in a large number of human tumor cell lines correlated with wild-type p53 status [37]. In addition, a few reports suggested p53-independent activities for Hdmx. For example, Hdmx has been implicated to suppress transcriptional activity of E2F1 [38] and Smad proteins [39;40], and to downregulate p21 protein levels [41]. However, p53 remains its major cellular target. Since uveal melanomas usually harbor wild-type p53, a subset of these cancers probably relies on increased levels of Hdm2 or Hdmx. To investigate this, we evaluated the status of the p53 pathway in uveal melanoma, with particular focus on Hdmx. Interestingly, when performing functional analysis of Hdmx in several selected uveal melanoma cell lines we encountered a growth promoting function of Hdmx that is independent of p53 inhibition. Our findings suggest that a novel p53-independent function of Hdmx is relevant in uveal melanoma and that targeting Hdmx may be beneficial in a subset of these tumors.

Results

Hdmx is over-expressed in a subset of uveal melanomas

A panel of ten uveal melanoma cell lines was analyzed for basal levels of several key proteins in the p53 pathway (Figure 1a). The levels of p53 itself were found to be more or less constant in all cell lines and they were comparable to the wild-type p53 expressing osteosarcoma cell line U2OS that we used as control, suggesting the absence of p53 mutations. The double band pattern observed in some lanes most likely represents the p53 codon 72 polymorphism [44]. The levels of Hdm2 and Hdmx varied greatly between cell lines, with most cell lines showing increased levels of at least one of these proteins.

In addition to OCM8, especially the cell lines derived from a metastasis (Omm1, Omm2.3 and Omm2.5) show very low levels of Hdmx protein. Recently, we found that especially in later stage tumors the relative expression of an alternative splice variant of Hdmx, Hdmx-S is increased accompanied with lower Hdmx protein levels, correlating with lower survival of patients (Lenos et al., in preparation). Therefore, we investigated the levels of Hdmx and Hdmx-S mRNA in the panel of uveal melanoma cell lines. Indeed, the results revealed that all three metastasis-derived cell lines express relatively high levels of Hdmx-S mRNA (Figure 1b). We also analyzed Hdm2 and Hdmx protein levels in lysates of fresh-frozen uveal melanoma tumor tissue and compared these with normal uveal melanocytes (NUM),
High levels of Hdmx promote cell growth in a subset of uveal melanomas

OCM8, 92.1 and U2OS lysates, representing low and high level Hdm2 and Hdmx controls (Figure 1c and Supplementary Figure 1). Hdm2 levels were significantly elevated in 5 out of 23 tumor samples (22%) compared to NUM. In 7 samples (30%) we found increased levels of Hdmx; 3 of these tumor samples overlapped. These findings suggest that Hdm2 and Hdmx over-expression control p53 activity in a subset of uveal melanomas.

**Cell line-dependent growth inhibition upon Hdmx knockdown**

To investigate whether high Hdmx expression indeed contributes to the growth of uveal melanoma cell lines, we selected three cell lines from the panel based on their differential expression of Hdmx and Hdm2. The 92.1 cells express high levels of Hdmx and low Hdm2, Mel202 cells express high levels of Hdmx and moderate Hdm2, and Mel285 cells express
moderate levels of Hdmx and high Hdm2. We reduced Hdmx expression using shRNA and analyzed cell proliferation/survival. Hdmx knockdown strongly suppressed growth of both 92.1 and Mel202 cells, whereas the growth of Mel285 cells was largely unaffected (Figure 2). This difference in sensitivity is most likely the result of differences in Hdm2 levels, which are highest in Mel285 cells. We have previously shown that Mel285 cells are sensitive to Nutlin-3 treatment, precluding the argument that p53 is not wild-type in Mel285 cells [45].

**P53-independent growth inhibition upon Hdmx knockdown**

To investigate whether the growth inhibitory effect of Hdmx knockdown was p53-dependent, we generated stable shp53 and shCtrl cell lines, which were transduced with shCtrl or three different shHdmx RNAs (Figure 3a). Surprisingly, p53 depletion did not rescue the effects of Hdmx knockdown in a 5-day growth assay, for none of the knockdown constructs (Figure 3b). We further evaluated the biological effects of Hdmx depletion by flow cytometry and found a clear G1 arrest, which was largely p53-independent (Figure 3c). Notably, a proportion of cells did not arrest in G1 upon Hdmx knockdown, as visualized by BrdU incorporation (Supplementary Figure 2a, left). Analysis 16h after BrdU removal indicated that a fraction of shHdmx cells still managed to enter the cell cycle and replicated at similar rate compared to the shCtrl cells (Supplementary Figure 2a, middle). This could be due to incomplete Hdmx knockdown in this cell fraction. Indeed, we found a selection for cells in which the knockdown was weaker: after 5 weeks culturing under puromycin selection, Hdmx expression returned to normal levels (Supplementary Figure 2b) and proliferation was no longer affected (Supplementary Figure 2a, right). In addition to cell cycle arrest, loss of Hdmx also resulted in a modest increase of Sub-G1 fraction (Figure 3d)
High levels of Hdmx promote cell growth in a subset of uveal melanomas

Figure 3  p53-independent growth inhibition upon Hdmx knockdown. (a) 92.1 cells were stably transduced using shCtrl or shp53 RNAs. The resulting cell lines were transduced with shCtrl or with three different shHdmx RNAs, and protein extracts were analyzed by western blot using the indicated antibodies. (b) Cells were counted and seeded for WST-1 proliferation assay, and cell viability was measured after five days. (c) Stable 92.1-shCtrl and 92.1-shp53 cells were transiently transduced with shCtrl or shHdmx#1 RNA and after six days, cell cycle profiles were analyzed by flow cytometry. Bars represent the mean and s.e. of two independent experiments. (d,e) Evaluation of Sub-G1 fractions and Annexin V staining, four days after transduction. Bars represent the mean and s.e. of three independent experiments. Statistical analysis was performed using a two-tailed t-test. (f-h) Stable 92.1-shCtrl and 92.1-shp53 cells were transiently transduced with shCtrl or shHdmx#1 RNA and subsequently mock-treated or treated with 10 μM Nutlin-3 for 24h. (f) Protein extracts were analyzed by western blot using the indicated antibodies. (g) qRT-PCR analysis of the expression levels of Hdm2, PUMA, p21 and Gadd45α, normalized for the geometric mean of CAPNS1, GAPDH and ARP. (h) Cells were counted and seeded for WST-1 proliferation assay. Cells were mock-treated or treated with 10 μM Nutlin-3, and cell viability was measured at several time points during five days.
and of Annexin V staining (Figure 3e). These results indicate some increased apoptosis upon Hdmx knockdown, in part p53-dependent, although the inductions were borderline significant.

The above described effects of Hdmx depletion in shp53 cells would be easily explained if the knockdown of p53 would be far from complete. In that case, Hdmx knockdown would still lead to p53 reactivation and p53-dependent growth inhibition. Therefore, we tested the efficiency of p53 knockdown by treating the cells with Nutlin-3, a potent activator of p53 [46]. Nutlin-3 was designed to bind Hdm2, but it also binds Hdmx albeit with lower affinity [35;47]. As shown in Figure 3f, 24h Nutlin-3 treatment strongly induced the protein levels of p53 and its target genes Hdm2, PUMA and p21 in shCtrl cells. We also found inductions of Hdm2, PUMA, p21 and Gadd45α at the mRNA level (Figure 3g). Of note, Hdmx knockdown in shCtrl cells also affected the expression of p53 targets, indicating some p53 activation in response to Hdmx depletion in these cells. Nutlin-3 strongly suppressed cell proliferation, and the growth inhibiting effect of Hdmx knockdown was further enhanced (Figure 3h). Importantly, the shp53 cells showed strongly reduced effects of Nutlin-3 on protein levels, mRNA levels and cell proliferation, indicating that the p53 knockdown was indeed sufficient to prevent p53 activation by Nutlin-3. In addition, FACS analysis revealed that Nutlin-3 treatment in shCtrl cells caused G1 arrest and increased Sub-G1 fractions, and it further enhanced the effects of Hdmx knockdown, whereas these effects were largely absent in shp53 cells (Supplementary Figure 3). Importantly, we observed similar p53-independent effects of Hdmx depletion on protein levels, mRNA levels, proliferation, and cell cycle profiles in Mel202 cells (Supplementary Figure 4a-d). Together, these findings indicate that the growth inhibition upon Hdmx knockdown is at least in part due to a novel function of Hdmx that stretches beyond p53 inhibition.

Reducing the expression of the retinoblastoma gene fails to rescue growth inhibition upon Hdmx knockdown

To characterize the aforementioned p53-independent function of Hdmx, we investigated the involvement of a few obvious candidates. First, we reasoned that in addition to p53, also the p53 homolog p73 might be bound and inhibited by Hdmx. Release of this inhibition would then result in p73-dependent growth suppression. However, we have not been able to detect p73 protein in these cells (not shown), making it unlikely that p73 reactivation is responsible for the observed effects of Hdmx knockdown. Next, we investigated a putative involvement of the tumor suppressor protein Rb. We hypothesized that, similar to the reported activity of Hdm2 towards Rb [48], Hdmx may also function via
High levels of Hdmx promote cell growth in a subset of uveal melanomas

Figure 4  Rb knockdown fails to rescue growth inhibition upon Hdmx knockdown. (a) 92.1 cells were stably transduced with shCtrl, shp53, shRb or with a combination of shp53 and shRb RNAs. The four resulting cell lines were transduced with shCtrl or shHdmx#1 RNAs and after four days protein extracts were analyzed by western blot using the indicated antibodies. (b) Cells from (a) were counted and seeded for WST-1 proliferation assay, and cell viability was measured at several time points during five days.

Rb inhibition. We stably reduced Rb expression via shRNA expression (Figure 4a) and investigated the effect of Hdmx knockdown on proliferation (Figure 4b). We found that reduced Rb levels failed to rescue the growth suppression upon Hdmx knockdown, although in this particular experiment the Rb knockdown is not very effective (Figure 4a).

Analysis of Hdmx knockdown-induced changes in expression of apoptosis-related genes

To search for genes that contribute to p53-independent apoptosis induction, we used a RT² Profiler PCR Array, which analyses the expression of 84 genes involved in apoptosis. Six genes showed more than 1.5 fold increased mRNA expression in both shCtrl and shp53 cells (Supplementary Figure 5a), of which four (BCL2L11, Caspase 4, Caspase 9 and TNFRSF1A) were annotated as apoptosis promoting genes. Seven genes showed more than 1.5 fold reduction of mRNA expression in both shCtrl and shp53 cells (Supplementary Figure 5b), of which three (Akt1, TRAF2 and BCL2L2) were annotated as apoptosis suppressing genes. Unfortunately, for none of these genes subsequent experiments could validate their expression to correlate with Hdmx knockdown (data not shown). This suggests that the p53-independent induction of apoptosis in response to Hdmx knockdown does not occur via transcriptional regulation, at least as far as the 84 genes represented on the array are concerned.
Chapter 3

Survivin over-expression fails to rescue growth inhibition upon Hdmx knockdown

Interestingly, Hdmx knockdown reduced the mRNA expression of the inhibitor of apoptosis (IAP) family member Survivin, which was not included in the RT² Profiler PCR Array (Supplementary Figure 6a). This reduction was partially p53-dependent as indicated by the effects of Nutlin-3. However, there may also be a p53-independent effect, as the Survivin expression was also somewhat reduced upon Hdmx knockdown in the shp53 cells. At the protein level, we found similar effects of Hdmx knockdown, using two shRNAs targeting Hdmx (Supplementary Figure 6b). We investigated a putative contribution of Survivin to the growth inhibiting effect of Hdmx knockdown by stable Survivin over-expression. However, despite high levels of exogenous Survivin (Supplementary Figure 6c), this could not rescue the negative effects of Hdmx knockdown on proliferation (Supplementary Figure 6d).

Induction of p27 protein levels upon Hdmx knockdown occurs independently of p53 and contributes to G1 arrest

The G1 arrest that is induced by Hdmx knockdown could be caused by increased levels of Cdk inhibitor(s). For example, p21 has been reported to interact with Hdmx, which leads to p21 degradation [41]. However, we found strongly reduced mRNA and protein levels of p21 in shp53 cells as compared to shCtrl cells (Figure 3f and 3g and Supplementary Figure 4a and 4b). Especially since the p21 levels in shp53-shHdmx cells were much lower than in shCtrl cells, a functional contribution of p21 to the observed effects of Hdmx knockdown is highly unlikely.

Another Cdk inhibitor that might be involved in the growth inhibiting effects of Hdmx knockdown is p27. Interestingly, p27 is a transcriptional target of FOXO proteins, and Hdm2 has been reported to inhibit activity of FOXO proteins by inducing the poly-ubiquitination of FOXO1 and FOXO3A [49] and mono-ubiquitination of FOXO4 [50]. Therefore, Hdmx might also function via regulation of FOXO proteins. Indeed, we observed an induction of p27 protein levels after Hdmx knockdown in 92.1 (Figure 5A) and Mel202 cells (Supp Figure 4A), irrespective of p53 levels. However, p27 mRNA was not induced (Figure 5B) indicating that the increased p27 levels in response to Hdmx knockdown does not occur at the transcriptional level. Importantly, we found p27 protein induction already one day after Hdmx knockdown (Figure 5C), a time frame during which the G1 arrest is not yet maximal (not shown), suggesting that the increased p27 protein level is not a secondary effect of cell cycle arrest [51-53]. To further examine the relevance of p27
High levels of Hdmx promote cell growth in a subset of uveal melanomas

induction in the responses to Hdmx knockdown, we reduced p27 expression by shRNA (Figure 5A) and analyzed cell cycle progression. Interestingly, the reduction of cells in S-phase (Figure 5D) as well as the amount of BrdU positive cells (Figure 5E) upon Hdmx knockdown was partially rescued by p27 depletion. A summary of these experiments (Figure 5F) illustrates that p27 knockdown partially prevents the G1 arrest induced by Hdmx knockdown, on top of a partial rescue by p53 depletion.

**Figure 5** Induction of p27 protein levels upon Hdmx knockdown occurs independently of p53 and contributes to G1 arrest. A, 92.1 cells were stably transduced with shCtrl, shp53, shp27 or with a combination of shp53 and shp27 RNAs. The resulting cell lines were transduced with shCtrl or shHdmx#1 RNAs and after four days protein extracts were analyzed by western blot using the indicated antibodies. B, Cells were transduced as indicated and analyzed by qRT-PCR for p27 expression levels, normalized for the geometric mean of CAPNS1, ARP and RPS11. C, 92.1-shp53 cells were transduced with shCtrl or shHdmx#1 RNAs. Protein extracts were isolated at the indicated time-points and analyzed by western blot using the indicated antibodies. D, Cells transduced as in A were analyzed by flow cytometry. E, Cells transduced as in A were incubated with 20 μM BrdU for 2 hrs and analyzed by flow cytometry. F, Quantification of D and E. Graphs indicate the fold reductions upon Hdmx knockdown of S-phase cells (mean and s.e. of two independent experiments) and BrdU positive cells.
Chapter 3

Discussion

Hdmx over-expression is found in a subset of human cancers, generally correlating with the presence of wild-type p53 protein [34-37]. Constitutive Hdmx over-expression contributes to the oncogenic transformation of cultured cells, thereby functionally resembling loss of p53 [34;54]. These findings emphasize that Hdmx over-expression in cancer mainly serves to block p53 activity. Indeed, in this study we show Hdmx over-expression in a subset of cell lines and fresh-frozen tumor samples from uveal melanoma, which rarely contain p53 mutations. Increased levels of Hdm2 were also observed in some cell lines and tumor samples, although the extent of over-expression was not impressive when compared to normal uveal melanocytes. Interestingly, our experiments in uveal melanoma cell lines also suggest the existence of an additional growth promoting function of Hdmx. Of note, we used three different Hdmx knockdown constructs and observed comparable effects on proliferation in 92.1 and Mel202 cells, whereas Mel285 cells remained largely unaffected. This indicates that a subset of uveal melanomas depends on Hdmx over-expression. In addition, the resistance of Mel285 cells reduces the likelihood of non-specific effects caused by the Hdmx knockdown constructs in 92.1 and Mel202 cells. Importantly, the lack of growth inhibition by Nutlin-3 in shp53 cells confirmed the efficiency of the p53 knockdown, indicating that Hdmx promotes uveal melanoma growth partially through p53-independent pathways.

At first sight this finding is a little surprising, particularly in light of the complete rescue of the embryonic lethality of Mdmx deletion by loss of p53 [55;56], which would argue against the importance of p53-independent effects of Hdmx. On the other hand, the physiological role of basal Hdmx levels during development may not be identical to the pathological effects of Hdmx over-expression during tumorigenesis. In addition, it is becoming increasingly clear that Hdm2 activity, and especially pathologically high levels of Hdm2, is not restricted to p53 regulation. Because of the homology between Hdm2 and Hdmx, our search for the mechanisms underlying p53-independent activities of Hdmx was primarily based on known functions of Hdm2. Enhanced Hdm2 activity has been reported to inhibit Rb function via ubiquitin-dependent degradation [48]. The Rb tumor suppressor protein represses E2F1 transcriptional activity via direct protein-protein interaction. Once released from Rb, resulting from Cyclin-Cdk mediated Rb phosphorylations, E2F1 transcriptionally activates genes involved in G1-S transition. A putative inhibiting function of Hdmx towards Rb might, therefore, explain the growth suppressing effect of Hdmx knockdown, as loss of such inhibition would subsequently lead to Rb reactivation.
High levels of Hdmx promote cell growth in a subset of uveal melanomas

However, the lack of rescue in our Rb knockdown experiments suggests that Rb is not responsible for the observed effects of Hdmx knockdown.

Interestingly, our results point at p27 protein induction as being one of the factors contributing to the growth inhibiting effects of Hdmx knockdown. Although p27 may exert some functions that are potentially oncogenic, it is generally considered to be a tumor suppressor [57]. The main role of p27 is to regulate the G0/G1 to S transition by binding and inhibiting cyclin E/CDK2 and cyclin D/CDK4,6 complexes. P27 itself is highly regulated at multiple levels, including transcription, translation, phosphorylation and ubiquitination [57;58]. P27 protein levels are maximal during G0 and early G1, mainly due to differences in cap-independent translation [59] and ubiquitin-dependent proteolysis [60] in different stages of the cell cycle. However, the induction of p27 protein levels in response to Hdmx knockdown probably occurred too quickly to be a secondary event of the G1 arrest. Moreover, p27 knockdown partially prevented the G1 arrest in response to Hdmx knockdown. This indicates that Hdmx somehow prevents p27 from inhibiting cell proliferation, via an unknown mechanism. Interestingly, Hdm2 can target FOXO proteins [49;50] and FOXO proteins regulate p27. Therefore, a reduction of Hdmx might lead to increased activity of FOXO proteins towards p27. Although we detected no changes in p27 mRNA expression, p27 regulation by FOXO proteins may exceed transcription. For instance, FOXO4 inhibits Akt1 to promote p27 nuclear translocation [61], and FOXM1 increases p27 stability [62]. Therefore, a closer examination of the involvement of FOXO proteins might still be rewarding.

Our search for genes contributing to apoptosis induction upon Hdmx knockdown turned out to be disappointing. Overall, the changes in mRNA expression of the 84 genes on the profiler array were rather small, which on itself might fit with the rather modest apoptosis induction in response to Hdmx knockdown. However, none of the ‘hits’ could be validated in additional tests. Thus, either the responsible gene(s) were not represented on the array, or the induction of apoptosis is transcription-independent. We further looked into the inhibitor of apoptosis (IAP) family member Survivin, since it is aberrantly expressed in a variety of human cancers [63]. Furthermore, a few reports suggest that this also includes uveal melanoma. A comparative transcriptomic analysis of uveal melanoma and normal uveal melanocytes revealed an upregulation of BIRC5 (the gene encoding Survivin) [64], whereas another study reported elevated expression of Survivin in several uveal melanoma cell lines, including 92.1, correlating with enhanced cisplatin resistance [65]. However, Survivin over-expression did not affect the outcome of Hdmx knockdown experiments, despite the reduction of endogenous levels. Indeed, Survivin transcription is
regulated in a cell cycle-dependent manner, peaking at mitosis [66;67], so the reduced Survivin levels may have been an indirect effect of Hdmx knockdown-induced G1 arrest.

In conclusion, Hdmx over-expression is present in a subset of uveal melanomas, most likely to promote tumorigenesis by inhibiting p53, which is rarely mutated in this type of tumors. Interestingly, however, we show that Hdmx also has an important p53-independent role in promoting cell proliferation and survival. It will be important to analyze the relevance of this role of Hdmx in other cell types as well. Our attempts to uncover the molecular basis of a p53-independent function of Hdmx have revealed a contribution for p27 in the induction of G1 arrest. Future studies are required to provide more insights into the mechanism by which Hdmx affects p27 protein levels. However, our data strongly suggest the involvement of additional, yet unknown factors, although unraveling these factors thus far proved difficult. In this respect, it may be worthwhile to investigate the involvement of proteins reported to interact with Hdm2, but not tested in this study, since they might interact with Hdmx as well. Alternatively, a mass spectrometry screen for Hdmx binding partners and functional characterization of newly found interactions might open new avenues to clarify p53-independent activities of Hdmx. Together this will improve our understanding of Hdmx over-expressing tumors and ultimately may lead to the development of new therapeutic strategies to target such tumors.

Materials and Methods

Cell lines, lentiviral transductions, drug treatments
Human uveal melanoma cell lines 92.1 [42], Mel202 and Mel285 were cultured in RPMI + F10 medium (1:1 ratio) with 10% fetal bovine serum (FBS) and antibiotics. Lentiviral constructs (listed in Supp Table 1) were described before [43] or obtained from the Mission shRNA library (Sigma-Aldrich, St Louis, MO). For lentiviral transductions, cells were seeded at a density of 4.0*10^5 (92.1 and Mel285) or 6.0*10^5 (Mel202) cells per 6 cm dish. The next day, cells were transduced using MOI = 1.0 in medium containing 8.0 μg/mL polybrene and were puromycin-selected for stable expression. Nutlin-3 was used at a final concentration of 10 μM and was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Immunoblotting
Cells were lysed in Giordano 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, blotted onto Polyvinylidene Fluoride Transfer membranes, incubated with the appropriate primary (listed in
Supp Table 2) and secondary antibodies, and bands were visualized by chemoluminescence (West Dura, Pierce Biotechnology, Rockford, IL).

**RNA isolation, qRT-PCR**

RNA was isolated using the SV Total RNA isolation kit (Promega, Madison, WI). cDNA was synthesized using 2.0 μg RNA in a total volume of 30 μL 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 listed in Supp. Table 3.

**Flow cytometry**

Cells were harvested, washed in PBS and fixed in ice-cold 70% EtOH. Prior to FACS analysis, cells were washed in PBS and resuspended in PBS containing 50 μg/mL RNase A and 50 μg/mL propidium iodide (PI). Flow cytometry was performed in the BD LSR II system (BD Biosciences). For Annexin V staining, cells were washed twice in PBS and resuspended in Annexin V-binding buffer containing Fluorescein isothiocyanate (FITC)-labeled Annexin-V (Sigma-Aldrich) and PI. After 10 min RT incubation cells were analyzed by flow cytometry. Positive PI staining, indicating necrotic or late apoptotic cells, were excluded from the analysis. PI-negative, Annexin V-positive cells represent early apoptotic cells. For bromodeoxyuridine (BrdU) incorporation, we added BrdU to the culture medium at a final concentration of 20 μM for 2h. Cells were harvested, washed in PBS and fixed in ice-cold 70% EtOH. Subsequently, cells were treated with 50 μg/ml RNase A (30 min 37 °C), washed and resuspended in 5 M HCl / 0.5% Triton (20 min RT). Cells were then neutralized in 1 M Tris/HCl pH 7.5, washed in PBS and incubated with anti-BrdU-FITC antibody (50 μg 11 202 693 001, Roche) in PBS/Tween with 1% BSA (30 min RT). Cells were washed twice in PBS/Tween, resuspended in PBS containing 50 μg/mL PI and analyzed by flow cytometry to detect BrdU and PI staining.

**WST-1 proliferation assay**

Cells were counted and seeded in triplicate in 96-well plates at a density of 3000 (92.1 and Mel285) or 6000 (Mel202) cells per well, in a total volume of 100 μL culture medium. To determine the survival/cell growth, 10 μL WST-1 (Roche) was added to the wells and absorbance (450 nm) was measured 2 hrs later in a microplate reader (Victor; Perkin Elmer).

**Acknowledgements**

We thank Prof. B.R. Ksander for providing the Mel cell lines, Dr. A. Levine for providing anti-Mdm2 antibody and Martijn Rabelink for help with the shRNA viruses.
Chapter 3

Reference List


High levels of Hdmx promote cell growth in a subset of uveal melanomas


Chapter 3


High levels of Hdmx promote cell growth in a subset of uveal melanomas


Supplementary Figure 1  Hdmx is over-expressed in a subset of uveal melanomas. Protein extracts of 23 fresh-frozen uveal melanoma tumor samples were analyzed for Hdmx and Vinculin protein levels by western blot, in comparison with the levels in NUM (Normal Uveal melanocytes) and Ocm8 cells (low Hdmx levels), 92.1 and U2OS cells, (high Hdmx). Quantifications of these blots are shown in Figure 1b.

Supplementary Figure 2  The partial G1 arrest upon Hdmx knockdown is lost after several weeks of puromycin selection. 92.1 cells were transduced with shCtrl or shHdmx#1 RNAs and after four days they were incubated with 20 μM BrdU for 2h. Subsequently, cells were immediately harvested (a, left panel), or harvested 16h after replacing the culture medium with medium lacking BrdU (a, middle panel) and cells were analyzed by flow cytometry. In addition, one dish for each transduction was maintained and propagated under puromycin selection. After 5 weeks, the surviving cells were incubated with 20 μM BrdU for 2h and analyzed by flow cytometry (a, right panel). Protein extracts were isolated every week and analyzed by western blot for Hdmx and γ-tubulin levels (b).
High levels of Hdmx promote cell growth in a subset of uveal melanomas.

Supplementary Figure 3  p53 knockdown is sufficient to prevent Nutlin-3 induced growth inhibition. Stable 92.1-shCtrl and 92.1-shp53 cells were transiently transduced with shCtrl or shHdmx#1 RNA. Subsequently, cells were mock-treated or treated with 10 μM Nutlin-3 for 24h and analyzed by flow cytometry.
### Supplementary Figure 4  

**p53-independent growth inhibition upon Hdmx knockdown in Mel202 cells.** A, Mel202 cells were stably transduced using shCtrl or shp53 RNAs. The resulting cell lines were transduced with shCtrl or shHdmx#1 RNAs. Four days after transduction, cells were treated with 10 μM Nutlin-3 for 24h and analyzed by western blot using the indicated antibodies. B, Cells were transduced and treated as mentioned in A and RNA expression was analyzed by qRT-PCR. Expression levels of Hdm2, PUMA, and p21 were normalized for the geometric mean of CAPNS1, GAPDH, and ARP. C, Cells were transduced as mentioned in A, counted and seeded for WST-1 proliferation assay. Cells were mock treated or treated with 10 μM Nutlin-3, and cell viability was measured at several time points during five days. D, Cells were transduced and treated as mentioned in A and analyzed by flow cytometry.
High levels of Hdmx promote cell growth in a subset of uveal melanomas

Supplementary Figure 5  Analysis of Hdmx knockdown-induced changes in expression of apoptosis-related genes. Stable 92.1-shCtrl and 92.1-shp53 cells were transduced with shCtrl or shHdmx#1 RNAs and total RNA was extracted four days post-transduction. Gene expression of 84 genes involved in apoptosis (and five housekeeping genes for normalization; B2M, HPRT1, RPL13A, GAPDH and ACTB) was assessed using the RT² Profiler™ PCR Array (SABiosciences) according to the manufacturer's instructions. Two independent experiments were averaged and genes demonstrating a 1.5-fold or greater increase (A) or decrease (B) are shown.

Supplementary Figure 6  Survivin over-expression fails to rescue growth inhibition upon Hdmx knockdown. A, Stable 92.1-shCtrl and 92.1-shp53 cells were transduced with shCtrl or shHdmx#1 RNAs and analyzed by qRT-PCR four day post-transduction. Expression levels of Survivin were normalized for the geometric mean of CAPNS1, GAPDH and ARP. B, Stable 92.1-shCtrl and 92.1-shp53 cells were transduced with shCtrl, shHdmx#1 or shHdmx#3 RNAs. Four day post-transduction, protein extracts were analyzed by western blot using the indicated antibodies. C, Stable 92.1-shCtrl and 92.1-shp53 cells were transduced with an empty vector or with a Flag-tagged Survivin expression vector, and neomycin selected to obtain stable cell lines. The resulting cell lines were transduced with shCtrl or shHdmx#1 RNAs. Four days post-transduction, protein extracts were analyzed by western blot using the indicated antibodies. D, Cells from C were counted and seeded for WST-1 proliferation assay, and cell viability was measured after five days.
## Supplementary Table 1: List of shRNA sequences and TRCN numbers

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Oligo sequences / TRCN numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>shCtrl</td>
<td></td>
</tr>
<tr>
<td>shp53</td>
<td></td>
</tr>
<tr>
<td>shHdm3fl1</td>
<td></td>
</tr>
<tr>
<td>shHdm3f2</td>
<td></td>
</tr>
<tr>
<td>shHdm3f3</td>
<td></td>
</tr>
<tr>
<td>shRb</td>
<td></td>
</tr>
<tr>
<td>shp27</td>
<td></td>
</tr>
</tbody>
</table>

**Supplementary Table 2: List of antibodies**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name/cat. #</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP</td>
<td>9542</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
</tr>
<tr>
<td>Hdm2</td>
<td>4B2</td>
<td>Chen et al., 1993</td>
</tr>
<tr>
<td>Hdm2</td>
<td>CEP14 0660</td>
<td>Enzo Life Sciences, CA, USA</td>
</tr>
<tr>
<td>Hdmx</td>
<td>A300-287A</td>
<td>Bethyl Laboratories, Montgomery, TX, USA</td>
</tr>
<tr>
<td>p-p53 Ser46</td>
<td>2180-1 / EP28Y</td>
<td>Epitomics, CA, USA</td>
</tr>
<tr>
<td>p-p53 Ser15</td>
<td>9284</td>
<td>Cell Signaling Technology, Beverly, MA, USA</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>PAb1801 / sc-98</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>PUMA N-terminal</td>
<td>P4743</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>p21</td>
<td>CPT4 / 05-655</td>
<td>Upstate Biotechnology, Lake Placid, NY, USA</td>
</tr>
<tr>
<td>RB</td>
<td>G3-245 / 554136</td>
<td>BD Pharmlingen, Franklin Lakes, New Jersey, USA</td>
</tr>
<tr>
<td>E2F1</td>
<td>K195 / sc-251</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>p27/Kip1 (C-terminal)</td>
<td>1591-1</td>
<td>Epitomics, CA, USA</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>TUB1 / 01087</td>
<td>Cell Signaling Technology, Beverly, MA, USA</td>
</tr>
<tr>
<td>γ-Tubulin</td>
<td>GTU-68 / T6557</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>Vinculin</td>
<td>hVIN-1 / V9131</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>HAUSP USP7</td>
<td>A300-033A</td>
<td>Bethyl Laboratories, Montgomery, TX, USA</td>
</tr>
</tbody>
</table>

For detection of human hMDM2 we used a mix of 4B2 and SMP14 (*), for detection of human p53 we used a mix of DO-1 and 1801 (*).


## Supplementary Table 3: Primer sequences used for qRT-PCR reactions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDM2</td>
<td>5′-ACGCAAGAAGCTTTCTCTATCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>PUMA</td>
<td>5′-CTGTACGAGATACAGAATTCACAG-3′</td>
<td>5′-AGAAGAATTCAGCTTCTCTACAG-3′</td>
</tr>
<tr>
<td>p21</td>
<td>5′-AGAAGAATTCAGCTTCTCTACAG-3′</td>
<td>5′-AGAAGAATTCAGCTTCTCTACAG-3′</td>
</tr>
<tr>
<td>GADD45a</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>p72</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>Survivin</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>CAPN1</td>
<td>5′-AGAAGAATTCAGCTTCTCTACAG-3′</td>
<td>5′-AGAAGAATTCAGCTTCTCTACAG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AGAAGAATTCAGCTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>ARP</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>RPS11</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>Hdmx exon 3</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
<tr>
<td>Hdmx exon 8</td>
<td>5′-ACGCAAGAAGCTTTCTCTCTCTCTCT-3′</td>
<td>5′-TCGAGATACAGAATTCACAG-3′</td>
</tr>
</tbody>
</table>