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Chapter 6

General Discussion

It has been long recognized that the p53 tumor suppressor protein plays a key role in cancer and its direct or indirect inactivation is an almost universal feature of human tumors. Collective studies in the last decades have resulted in a profound understanding of p53's regulation and function, and p53 is now placed in a central position in the prevention of genomic instability and protection of tumorigenesis, therefore nicknamed "guardian of the genome" [1]. Clinical application of the obtained knowledge about p53 appears promising; however, there are still many challenges that remain to be addressed. This thesis has essentially focused on two different arms in p53 research, one involving the role of the p53 regulator Hdmx in cancer development (chapter 2 and 3) and the second exploring several aspects of the use of small-molecule p53 activators as cancer treatment (chapter 4 and 5).

Hdmx in oncogenic transformation

Designing future anticancer strategies that target the p53 pathway requires a detailed understanding of the functional properties of Mdm2 and Mdmx (in humans named Hdm2 and Hdmx) and the mechanisms controlling them. Hdm2 is mostly regarded the more general regulator of p53, with Hdmx being its little brother. This is exemplified by the notions that unlike Hdm2, Hdmx on its own cannot degrade p53 or facilitate its nuclear export [2;3]. Hdmx blocks p53 transcriptional activity and may serve as a cofactor for Hdm2-mediated p53 ubiquitination. Furthermore, Hdmx loss as compared with Hdm2 loss generally appears to exhibit less severe effects, in terms of the timing of embryonic lethality in mice [4-6] as well as the toxicity in adult tissues [7;8]. In addition, Mdm2 overexpression rescues the lethal phenotype of Mdmx knockout in mice [9], whereas widespread Mdmx overexpression appears not to compensate for Mdm2 loss [10]. Nevertheless, a subset of human cancers relies on Hdmx overexpression to inactivate the p53 tumor suppressor pathway [11-14]. This suggests that increased Hdmx levels are sufficient to counteract the oncogene-induced p53 activity during neoplastic transformation. Indeed, this has been shown for murine cells [11]. Importantly, chapter 2 of this thesis also provides evidence for such a direct role of Hdmx in cultured human cells. When combined with other defined genetic changes, constitutive Hdmx overexpression contributed to the oncogenic transformation of both foreskin fibroblasts (VH10) and human embryonic retinoblasts (HER). The choice for retinoblasts was based on the frequent Hdmx overexpression observed in retinoblastoma. Like most Hdmx overexpressing cancers, retinoblastoma retains wild-type p53. This suggests that the oncogenic function of Hdmx is based on p53 inhibition, which is supported by the observations in chapter 2 that cells with increased Hdmx levels functionally resembled the

p53 knockdown cells. The most transformed HER cells, with either Hdmx overexpression or p53-knockdown, also showed *in vivo* growth capacity, although limited.

The principal effector linking upstream oncogenic signals and p53 activation is the tumor suppressor protein p14^{ARF}, a direct target of the Rb-repressed transcription factor E2F1, which antagonizes Hdm2 function [15-17]. This pathway may be particularly relevant in retinoblastomas, which harbor direct Rb gene inactivation. Importantly, p14^{ARF} does not directly prevent p53 inhibition by Hdmx [18], although it has been proposed that p14^{ARF} stimulates Hdm2-mediated Hdmx degradation [19]. This suggests that in p14^{ARF}-proficient tumor cells, Hdmx has the capacity to bind and inhibit p53 without the help of functional Hdm2, but only when being sufficiently abundant. Hdmx overexpression does not reduce p53 protein levels, which is indeed observed in the transformed fibroblasts and retinoblasts in chapter 2. However, Hdmx effectively inhibited p53 activity, visualized by reduced basal mRNA and protein levels of p53 targets, except for Hdm2 protein, which is directly stabilized by Hdmx.

Hdmx in uveal melanoma

A proportion of human cancers contains increased Hdmx expression, mostly correlating with wild-type p53 status [11-14]. Chapter 3 investigated the role of Hdmx in uveal melanoma and showed Hdmx overexpression in a subset of cell lines and fresh-frozen tumor samples. Proliferation of some of these cell lines depended on Hdmx expression, as Hdmx reduction resulted in growth inhibition. Since uveal melanomas rarely contain p53 mutations, these cells most likely selected for high Hdmx levels because of its capacity to control p53 activity. Interestingly, however, the experiments in uveal melanoma cell lines indicated the existence of an additional growth promoting role of Hdmx. Such a p53-independent function of Hdmx may only become apparent when being overexpressed during tumorigenesis and not in normal development, as p53 deletion is known to prevent the effects of Mdmx knockout in mouse embryos [4-6]. The results in chapter 3 suggested the involvement of the Cdk inhibitor p27. Hdmx affected p27 at the post-transcriptional level, although the exact mechanism remains to be determined. Depletion of p27 partially rescued the induction of G1 arrest in response to Hdmx knockdown. The supposed p53-independent activity of Hdmx probably involves additional factors, although other contributors besides p27 could not yet be identified, including p73, Rb, p21, Survivin and a range of genes presented on an apoptosis-specific gene expression array. Therefore, further investigations are required to uncover the molecular basis of a p53-independent function of Hdmx in uveal melanoma. In addition, it is important to analyze the relevance

of such a role of Hdmx in other cell types as well. This will teach us more about Hdmx overexpressing tumors and ultimately may result in novel strategies to target such tumors.

Effect of Hdmx on the responses to Nutlin-3 treatment

The development of specific drugs that reactivate p53 in tumor cells is a promising strategy to treat cancer. A well-studied example is the non-genotoxic Hdm2 antagonist Nutlin-3, which disrupts the Hdm2-p53 interaction and has already shown therapeutic potential in both *in vitro* and *in vivo* experiments [20]. The affinity of Nutlin-3 for Hdmx is much lower as compared to Hdm2 [12;21]. It has been argued that Hdmx levels dictate a cells' sensitivity to Nutlin-3 [22-24], because high levels of Hdmx may still be capable of binding and inhibiting p53 even in the presence of Nutlin-3. The established transformation models in chapter 2 provided a nice opportunity to investigate the effects of Hdmx overexpression on the outcome of Nutlin-3 treatment, in addition to assessing the role of Hdmx in neoplastic transformation. Indeed, Hdmx overexpression in skin fibroblasts was sufficient to attenuate p53 activation and growth inhibition mediated by Nutlin-3. However, Hdmx overexpression in HER cells could not prevent p53 induction and growth inhibition by Nutlin-3. Most likely, the lower Hdmx levels in the HER cells underlie this difference between VH10 and HER cells. Upon Nutlin-3 treatment, Hdmx levels were further reduced, due to Hdm2-mediated degradation. Retinoblastoma cell lines also show sensitivity to Nutlin-3, despite the presence of high Hdmx levels [12], indicating that the transformed HER cell lines provided a physiologically relevant model. Chapter 3 and 4 showed that Nutlin-3 efficiently induces p53-dependent growth inhibition in uveal melanoma cell lines. Interestingly, cell lines expressing high (92.1 and Mel202) and low levels of Hdmx (Mel270 and Mel285) displayed similar sensitivity to Nutlin-3. Thus, the results in HER cells and uveal melanoma cell lines suggest that pathological Hdmx expression *per se* is not an all determining single determinant for the efficacy of Nutlin-3 treatment.

Use of Nutlin-3 in combination treatments

Nutlin-3 alone is probably not sufficient as cancer treatment, since in many wild-type p53 expressing tumors the Nutlin-3-activated p53 mainly leads to a reversible cell cycle arrest. Obviously, induction of apoptosis would lead to a more effective eradication of tumor cells. Chapter 4 investigated whether the therapeutic potential of Nutlin-3 could be increased by combination with other drugs. Calculated combination index values [25] demonstrated that Nutlin-3 synergized with both the topoisomerase I inhibitor Topotecan and with the small-molecule p53-activator RITA [26] to inhibit uveal melanoma growth,

correlating with enhanced apoptosis induction. Growth inhibition by Nutlin-3 and Topotecan was confirmed *in vivo* in a mouse model for ocular melanoma [27]. Importantly, low oxygen levels, often occurring *in vivo*, were found to not interfere with the efficacy of the proposed treatments. Thus, chapter 4 holds a strong case for using small-molecule p53 activators in combination therapy as treatment for ocular melanomas.

DNA damage response and p53-Ser46 phosphorylation

Chapter 4 also describes attempts to find a mechanistic explanation for the enhanced induction of apoptosis when combining Nutlin-3 with Topotecan or RITA. In chapter 5, the focus gradually shifted toward the mechanistic properties of RITA alone, which appeared to be very complex (discussed below). The increased p53-Ser46 phosphorylation as observed in both combination therapies was remarkable. This particular modification has been reported to contribute to directing the p53 response to apoptosis induction. The use of PIKK inhibitors and knockdown constructs against ATM in chapter 4 demonstrated that p53-Ser46 phosphorylation is a result of DNA damage signaling. Both Topotecan and RITA are known to induce a DNA damage response; Topotecan via inducing double strand DNA breaks and RITA via a poorly understood p53-dependent mechanism. However, ATM inhibition as described in chapter 4 and 5, in order to reduce DNA damage signaling and p53-Ser46 phosphorylation, on itself appeared to affect cell viability, and no rescue of apoptosis could be observed. Therefore, a functional contribution of the DNA damage response in general and p53-Ser46 phosphorylation in particular to the enhanced induction of apoptosis by the combination treatments remained unclear. An additional experiment in chapter 5, which focuses on RITA and no longer on Topotecan, further questioned the relevance of DNA damage signaling for apoptosis induction. Serum starvation partially prevented the induction of apoptosis by RITA, but not the DNA damage response. Therefore, the DNA damage response may just be an irrelevant side effect of RITA treatment.

Role of Chk2 in mediating the responses to RITA

Despite the fact that both Nutlin-3 and RITA were identified as p53-activating molecules, they exhibit very different effects on cell cycle progression (G1 arrest vs. S and G2 arrest). This may be an important contribution to the observed synergy, as only a stronger activation of the same pathway would probably at best be additive, but not synergistic. It is becoming increasingly clear that RITA has a much broader impact than just stabilizing p53, which is best shown by the reported p53-dependent induction of DNA damage response

[28-31]. Indeed, the proposed mechanism, i.e. RITA binds directly to p53 and disrupts the p53-Hdm2 interaction, is controversial [32]. The necessity of Chk2 for the RITA-induced responses, presented in Chapter 5, adds another level of complexity. Chemical inhibition of Chk2 kinase activity in uveal melanoma cell lines provided the initial clue for a contribution of Chk2 to the responses to Nutlin-3 plus RITA. Similar to ATM inhibition, inhibiting Chk2 using drugs or knockdown reduced p53-Ser46 phosphorylation, suggesting the classical activation of Chk2 function as part of the DNA damage response. However, the RITA-induced DNA damage response itself appeared to be Chk2-dependent, indicating that Chk2 acts upstream of ATM. Moreover, the findings in chapter 5 indicated that Chk2, unlike ATM, has a crucial contribution to the biological effects of RITA. In fact, Chk2-null HCT116 cells largely mimicked the p53-null HCT116 cells in their RITA response: lack of replication stalling, DNA damage response, apoptosis induction, reduction of Hdm2 and Hdmx protein levels, full p53 stabilization and transcriptional regulation of p53-responsive genes. These data place Chk2 in a central position in mediating the cellular responses to RITA.

Although chapter 5 represents an important step forward with regard to the understanding of RITA's mechanism of action, many questions remain unanswered. First, it would be intriguing to find out in detail how RITA triggers p53 and Chk2 activity. The data in chapter 5 could implicate that p53 stabilization is partially indirect, via Hdm2 degradation. Identifying additional players that directly interact with RITA might provide important new insights. An early study investigating RITA (initially named NSC-652287) suggested the induction of both DNA-protein and DNA-DNA cross-links, correlating with cytotoxicity [33]. In addition, RITA was reported to bind multiple, not further characterized proteins and its differential cytotoxic activities in different cell lines were attributed to differences in cellular drug accumulation and the cell's capacity to metabolize RITA into reactive species [34]. Fitting these observations with later findings that RITA selectively kills transformed cells in a p53-dependent fashion [26] is a major challenge. Interestingly, chapter 5 suggested that Chk2 is essential for efficient activation of a previously reported p53-dependent S-phase checkpoint by RITA, involving Chk1 [28]. The induction of crosslinks may be relevant in this respect, however the nature of this checkpoint remains enigmatic and needs to be elucidated. In addition, it will be important to identify the substrate(s) of Chk2 that are relevant for the observed effects. Lastly, it is unclear whether Chk2 phosphorylation contributes to apoptosis induction by RITA, or whether this is a secondary effect of DNA damage signaling, resulting from replication stalling. Of note, the DNA damage response triggered by Nutlin-3 plus Topotecan (chapter 4) may be more relevant for the biological effects, as the induction of DNA damage signaling by Topotecan was much stronger as compared with RITA when the drugs were used at around IC50

concentrations. On the other hand, Chk2 inhibition also diminished apoptosis induced by Nutlin-3 plus Topotecan. This indicates that Chk2 may have an important role in translating replicative stress into an apoptotic response.

Future directions

Rapid advances are currently being made in drug-development aiming at reactivation of the p53 pathway [35]. Analogs of the Hdm2-antagonists Nutlin-3 and MI-219 have progressed to advanced preclinical development or early phase clinical trials [36]. A key issue for p53-activating anti-cancer strategies that still requires better understanding involves how to achieve a maximal apoptotic response in cancer cells and a mild, reversible growth arrest in normal tissues. The realization that a subset of cancers relies on Hdmx overexpression to inactivate p53, including retinoblastoma and uveal melanoma, may have important implications for the treatment of those tumors. Whereas Hdm2 inhibition may trigger pathological p53 function in normal tissues, the targeting of Hdmx will probably have a milder impact [7], thereby possibly increasing the therapeutic window of specific Hdmx inhibitors. Indeed, such compounds are now being developed [37]. As discussed in this thesis, the use of specific drug combinations could also be an effective method to increase the therapeutic efficacy of p53 activating drugs while reducing the toxic effects of the individual compounds. However, this will require a labor-intensive determination of a fixed ratio of the different compounds to work optimally together taking into account the variable pharmacological properties of the individual molecules. Combination therapy using non-genotoxic activation of p53 might also be useful in what is named cyclotherapy. In such a strategy, normal cells undergo a protective cell cycle arrest, whereas mutant-p53 expressing cells continue to proliferate and are more sensitive to subsequent treatment with anti-mitotic agents [38]. The use of RITA will be particularly useful in tumors harboring wild-type p53 and functional Chk2. However, a better understanding of RITA's molecular mode of action will help defining the optimal conditions for its efficient application in patients. Eventually, this will bring us closer to developing personalized and effective cancer treatments with reduced toxicity.

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