Novel functions of MDMX and innovative therapeutic strategies for melanoma

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CHAPTER 1

General introduction

1. THE P53 PROTEIN AND ITS REGULATORS MDM2 AND MDMX

1.1 p53

The p53 protein was originally discovered in 1979 as a target of the SV40 oncogenic DNA virus Large T-antigen [1, 2]. More than 3 decades later and over 92.000 scientific papers published mentioning p53, the p53 protein is recognized as a central node in cellular stress responses. The p53 protein functions as a transcription factor, which upon activation and stabilization controls the expression of many genes involved in multiple pathways including cell cycle, metabolism, apoptosis and angiogenesis [3-5]. Despite its central role in cellular responses to stress, p53-deficient mice develop almost normal, but are prone to develop malignancies of which lymphomas are most frequent [6, 7]. Mutations in the *p53* gene are found in proximally 50% of all human cancers, emphasizing the importance of the tumor suppressor function of p53 [8, 9]. A more detailed analysis shows that 95% of *p53* mutations are found in the exons encoding DNA binding domain, underlining its tumor suppressor function as transcription factor [10]. A mutation in the DNA binding domain renders p53 incapable of binding to its consensus DNA recognition sequence, losing its transcription regulatory function, rendering a cell sensitive for a malignant transformation and relatively resistant to stress induced apoptosis, cell cycle arrest or senescence, e.g. induced by chemotherapeutics, radiation or hypoxia.

Despite this high mutation frequency, incidence of *p53* mutations differs considerably between cancer types. *P53* mutations are found rarely (<1%) in, for example, uveal melanoma (UM) and thyroid cancer, while mutations are found commonly (>90%) in ovarian cancer and lung squamous cell carcinoma (Figure 1) [11]. It is believed that in tumors expressing wild-type p53 the tumor suppressor pathway of p53 is inhibited either upstream or downstream, implicating that all cancers have an attenuated p53 pathway [4].

1.2 Regulation of p53 by MDM2 and MDMX

The central and important functions of p53 in cell-fate determination imply that p53 activity should be tightly controlled, in which ubiquitin ligase mouse double minute (MDM) 2 and the structurally related MDMX play a pivotal role. The importance of the MDM2 and MDMX proteins for p53 regulation is best illustrated by the mouse KO models. Knockout of either *MDM2* or *MDMX* is embryonic lethal in a fully p53-dependent manner [12-14]. Whereas *MDM2* transgenic mice can rescue the *MDMX* knockout phenotype, high *MDMX* levels in *MDMX* transgenic mice cannot rescue the *MDM2* knockout [15, 16]. Although both *MDM2* and *MDMX* are crucial for embryonic development, in adult cells and tissues *MDM2* loss is still always lethal whereas

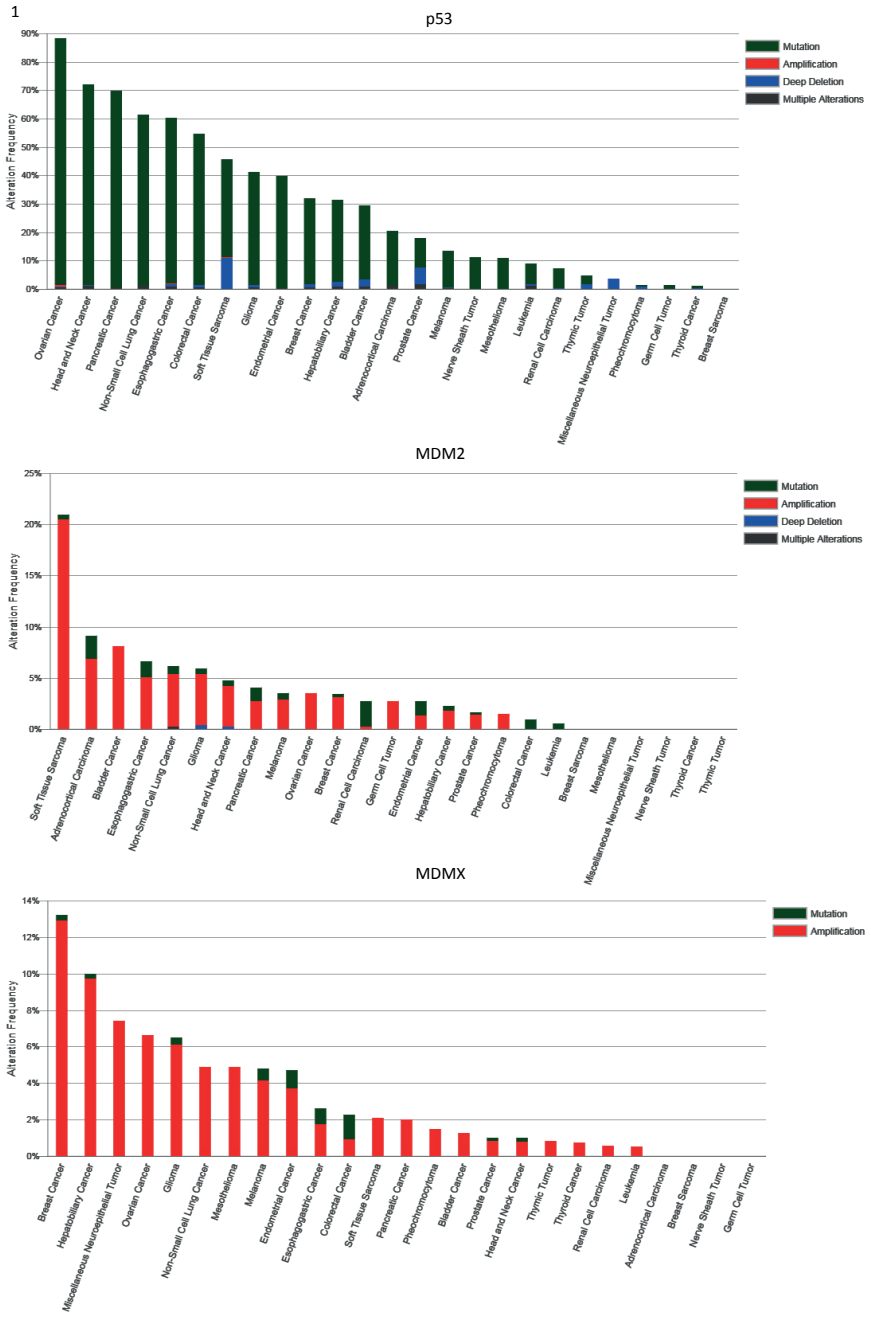

Figure 1. Genomic alterations affecting p53, MDM2 or MDMX in different cancers. Frequency of mutations (green), amplifications (red), deed deletions (blue) and multiple alterations (gray) are given per cancer. Data depicted is derived from www.cbioportal.org and only shows TGCA provisional data sets.

MDMX loss can be compatible with life, probably because in most adult tissues MDMX protein is not or hardly detectable anyway [17-23]. MDM2 is an E3 ubiquitin ligase and has been shown to directly bind p53 [24]. MDM2 activity results in lysine-48 poly-ubiquitination of p53, which is consequently degraded by the proteasome [25]. Thereby MDM2 effectively keeps the basal levels of p53 low and thus promotes cell proliferation and survival. Both the RING finger domain and the central acidic domain of MDM2 are essential for the p53 ubiquitination [26, 27]. Although MDM2 during animal development is mainly acting through the repression of p53, MDM2 has been reported to have p53-independent functions and ubiquitination targets [28-31].

The essential p53 inhibitor MDMX was initially discovered as a novel p53 interactor with high sequence homology with MDM2 [32]. MDM2 and MDMX have great structural similarities of which the N-terminal hydrophobic pocket that binds the N-terminal alpha helix of p53, shielding the p53 transactivation domain, is best conserved [33, 34]. Despite the high conservation of the RING finger domain and the central acidic domain MDMX does not have any E3 ubiquitin ligase activity and its main p53 inhibitory function is shielding the p53 transactivation domain [26, 27]. Despite the lack of intrinsic E3 ligase activity MDMX forms a heterodimer with MDM2 [35], which is thought to promote MDM2 E3 activity by providing a better scaffold for E2-enzyme binding, thus resulting in faster degradation of p53 [36, 37]. Considering that the levels of MDM2 and MDMX are crucial for cellular activity of p53, expression of these proteins should also be tightly controlled. P53 has to be liberated from MDM2 and MDMX to exert is function upon certain stress, for example in response to DNA damage. Several phosphorylation events on MDM2, mediated by serine/threonine kinase ATM, inhibit its ubiquitin ligase activity towards p53 [38]. Upon stresses, MDM2 both auto-ubiquitinates [38, 39] and ubiquitinates MDMX [40-42] sending both for proteasomal degradation. This cellular depletion of inhibitory proteins results in a feed forward loop in which p53 is stabilized and activated. After cellular stress, for example induced by DNA damage, during the recovery phase a cell needs to re-constrain p53. It has been shown that both MDM2 and MDMX are transcriptional targets of p53 providing a negative feedback loop and thus re-establish p53 inhibition [43, 44].

1.3 Reactivating p53 in cancer

In order to become malignant cells need to lose or at least attenuate p53 activity, for example by direct gene mutation [8, 9]. Therefore, specifically targeting p53 mutated cancer cells would provide a very interesting therapeutic intervention, potentially benefitting half of all cancer patients. It was reasoned that cancer cells with mutated p53 would remain sensitive for p53-induced apoptosis, since the downstream path-

way remains intact [45]. Therefore, various approaches were designed to reactivate mutant p53 [46]. One compound discovered to reactivate mutant p53 was named p53 reactivation and induction of massive apoptosis (PRIMA) [45], which binds the core domain the DNA binding domain of p53 and changes the conformation from mutant to wild-type, resulting in the induction of apoptosis [47, 48]. This biological effect induced by PRIMA has been suggested to be specific for p53 mutant cell lines [49]. However, evidence is accumulating that PRIMA induces anti-cancer effects regardless of the presence of p53 mutations [50, 51]. This could be explained, at least in part, by the observation that PRIMA also targets other p53 family members such as p63 and p73 [51-53]. Other approaches found to target p53 mutated cells include the cholesterol lowering drugs, the statins [54, 55]. Depletion of cells from mevalonate-5-phosphate by treatment with statins resulted in impairment of the mutant-p53 interaction with the chaperone protein DNAJA1/hsp40 which caused ubiquitin E3 ligase CHIP-mediated degradation of mutant p53 [55]. These studies have provided new insights with potential new strategies to specifically target mutant p53 cells.

Despite the frequent occurrence of *p53* mutations, the remaining half of human cancers had to find alternative mechanisms to attenuate p53 signaling [4]. Amplifications of the *MDM2* gene are frequently found in sarcoma [56-58] and esophageal cancer [59] (Figure 1). Similarly to *MDM2*, *MDMX* amplifications and overexpression are found in various cancers including glioblastoma [60], retinoblastoma [61] and breast cancer [62], in most cases correlating with wild-type *p53* status (Figure 1). The MDM2 interaction with the p53 transactivation domain is well defined by crystal structures [63]. These structures show that the hydrophobic pocket of MDM2 interacts with 3 side chains from a peptide derived from the p53 transactivation domain. This clearly defined pocket and interaction between MDM2 and p53 allowed for effective drug development. The first small molecule compound described to bind MDM2 in its p53-binding pocket was Nutlin-3 [64]. Antagonizing the MDM2-p53 interaction using Nutlin-3 resulted in stabilization of p53 in an MDM2-amplified osteosarcoma cell line, leading in cell cycle arrest and apoptosis, both *in vitro* and *in vivo*. Importantly, the p53 activation by Nutlin-3 was not due to DNA damage signaling [65, 66]. This mode of action resulted in the observation that mice treated with Nutlin-3 did not lose weight while p53 was being activated, indeed separating Nutlin-3 from DNA damaging agents and their associated adverse clinical effects [64]. This approach has spurred the development of various small molecule compounds targeting the MDM2-p53 interaction such as 1, 4-benzodiazepin-2, 5-dione [67], spiro-oxindoles [68] and RITA [69], all resulting in p53 stabilization and inducing cell cycle arrest and apoptosis. Although found in a screen to identify p53 re-activating compounds and thought originally to block the MDM2-p53 interaction, RITA elicits a DNA damage

response, rendering the anti-cancer effects not exclusive to the MDM2-p53 inhibition [70-72]. Furthermore, some evidence exists indicating that RITA does not block the MDM2-p53 interaction [73], implying that RITA targets cells expressing p53, but not by directly binding to p53.

Based on these promising results *in vitro* and in pre-clinical mouse models, a number of clinical trials were initiated using various compounds targeting the MDM2-p53 interaction [74]. RG7112, a Nutlin-3 analog, was initially tested in liposarcoma patients with *MDM2* amplifications. Of the 20 patients in this clinical trial 14 had stable disease and 1 patient had a partial response [75]. Besides its therapeutic potential RG7112 treatment elicited severe neutropenia and thrombocytopenia in these patients. In a phase 1 clinical trial assessing RG7112 in 116 patients with various hematological malignancies, similar to the sarcoma trial, 22% of the patients showed severe neutropenia [76]. Although MDM2 inhibition has a clinical benefit for these patients, the strong, on target, adverse effects need to be managed in order to continue long-term MDM2 inhibition [77]. In addition, resistance to MDM2 inhibition has been shown to occur via specific point mutations in *p53* [78, 79].

Antagonists for the MDMX-p53 interaction have been in development since *MDMX* amplification and/or overexpression in *p53* wildtype tumors was discovered. Despite the overall structural similarity between MDM2 and MDMX, some important differences were found in their p53 binding pocket [34, 80]. These slight changes in the p53-binding hydrophobic cleft reduce the binding capabilities of Nutlin-3 to MDMX approximately 40 fold, although Nutlin-3 can still clearly antagonize the interaction between MDMX and p53 [61]. The reduced efficacy of MDM2 inhibitors for MDMX suggested a window of specificity, which led to the pursue of an MDMX-specific inhibitor. SJ172550 was the first described small molecule specifically designed to block the MDMX-p53 interaction [81]. However, it has been described later that SJ172550 is not a simple inhibitor between MDMX and p53, but locks MDMX in a conformational state by covalent interaction that is unable to bind p53 [82]. Unfortunately, this conformational state change is dependent on many factors including the reducing potential of the media, hindering the further clinical development of SJ172550 [82]. Another study described molecules inhibiting MDMX transcription, e.g. XI-006 and XI-011 [83]. These compounds resulted in the cellular depletion of MDMX promoting p53 activation, reportedly without induction of double strands DNA breaks, providing treatment options for various cancers [84-86]. However, this MDMX depletion effect by XI-011 was later shown to be partly due to increased DNA damage signaling resulting in MDMX degradation and subsequent p53 activation [86, 87] and apoptosis induced by XI-006 in Ewing Sarcoma was even shown to be p53 independent [85]. It

thus appears that the design of small molecules specifically targeting MDMX without inducing DNA damage signaling is a difficult task. It could be that dual inhibitors of MDM2 and MDMX provide a solution [88]. By simultaneously inhibiting MDM2 and MDMX p53 activation is boosted, meaning that less MDM2 inhibition (and therefore less adverse effects) might be needed to achieve functional p53 activation.

Alternative approaches to target MDMX could involve other pathways, which have shown to play a role in overexpression of MDMX. It has been demonstrated that the receptor tyrosine kinases Her4 (also known as Erbb4) and AXL are capable of stabilizing MDMX in order to suppress p53 [89, 90]. Targeting these signaling pathways might be a potent way to destabilize MDMX, thus releasing p53 activity, possibly without inducing DNA damage signaling. However, these kinases have multiple targets and downstream effects independently of MDMX, which will make the analysis of these inhibitors on MDMX function especially difficult.

Alternative splicing is yet another mechanism by which the abundance of MDMX is reduced upon DNA damage [91]. The short isoform of MDMX, missing exon 6, is a naturally occurring transcript, which results in a short protein due to an early stop [92]. Mice that are lacking exon 6 are embryonic lethal in a p53-dependent manner [93]. By promoting the skipping of exon 6 using anti-sense oligonucleotides the splicing ratio could be altered favoring the short over the full length isoform, resulting in decreased MDMX protein levels [94]. MDMX has been shown to be a potent target in both melanoma [95] and wildtype p53 breast cancer [96]. Depletion of MDMX resulted in a cell cycle arrest and apoptosis in a partly p53-independent manner [87, 95]. The p53-independent cell cycle arrest could be explained, at least in part, by the p53-independent upregulation of the cyclin dependent kinase (CDK)-inhibitor p27 upon MDMX depletion [87]. These results suggest that MDMX might not only to be a therapeutic target in wildtype p53 tumors, but also in p53 mutated tumor cells. Indeed, p53 mutated breast cancer cell lines expressing high levels of MDMX are dependent on continuous MDMX expression for proliferation [97].

2. MELANOMA

To study the functions of p53 and especially of MDMX, this thesis focusses on melanoma, a malignancy arising from melanocytes. In cutaneous melanoma *p53* mutation frequency is low (10-20%) and UM cells essentially lack *p53* mutations [98, 99]. Despite the absence of *MDM2* or *MDMX* amplification, melanoma cells frequently overexpress one or both of these p53 inhibitors, especially MDMX [87, 95]. Since melanoma

patients with distant metastases respond poorly to classical chemotherapy and, therefore, have a short overall survival, studying melanoma with a focus on the MDMX/p53 complex is highly clinically relevant [100]. Although melanoma encompasses only a low percentage of skin cancer, melanoma is a deadly form of cancer causing most of the skin cancer-associated deaths. The increased melanoma incidence found over the last decades emphasizes the importance of finding an effective cure for melanoma [101]. Due to advances in early detection of melanoma the primary tumors can be efficiently resected resulting in high survival rates. However, prognosis significantly worsens upon metastasis. Improvements have been made during the past decades in understanding melanoma and how to use this knowledge to target this malignancy. The main current treatments for melanoma are briefly discussed below.

2.1 Cutaneous melanoma

2.1.1 Targeted therapy

Previous studies have already reported that the MAPK signaling pathway is activated in various cancer types including melanoma [102]. The most frequent and well described melanoma driver is an activating mutation in the serine/threonine kinase *BRAF* gene in up to 50% of melanomas. Most common mutation is the valine (V) substitution for glutamic acid (E) of codon 600 (V600E), feeding into the MAPK pathway and driving melanomagenesis [102]. Mutations upstream of *BRAF*, mainly in *NRAS*, are found in 10-25% of all cutaneous melanoma cases [103]. The most common activating *NRAS* mutation occurs at the codon for glutamine (Q) 61 [104]. These hotspot mutations in *BRAF* and *NRAS* rendering the proteins permanently active, and continuously stimulate the pro-proliferation MAPK pathway. Additionally, in 14% of cutaneous melanoma samples inactivating mutations are found in *NF1*, a GTPase-activating protein. By losing NF1 expression RAS-GTP is much slower converted to its inactive GDP form, resulting in increased RAS activation and consequently an overactive MAPK pathway. Therefore, loss of NF1 (14%), activating mutation in NRAS (28%) or in BRAF (52%) explains the activated MAPK signaling in 94% of all melanoma cases (Figure 2A) [104].

Recently, a novel classification was presented identifying four major subtypes of cutaneous melanoma; BRAF, NRAS, NF1 and the so called triple-negative [104]. Interestingly, mutations in the gene encoding the receptor tyrosine kinase (RTK) *KIT* are enriched in the triple-negative subgroup. Although only 3% of all melanoma have *KIT* mutations or amplifications, these mutations are more commonly found in melanoma originating from mucosal, acral a chronically sun-damaged surface [105]. Like *BRAF* and *NRAS*, mutations in *KIT* focus on a 'hot-spot' with 30% of *KIT* mutations showing an activating L576P substitution, suggesting a potential therapeutic benefit of the

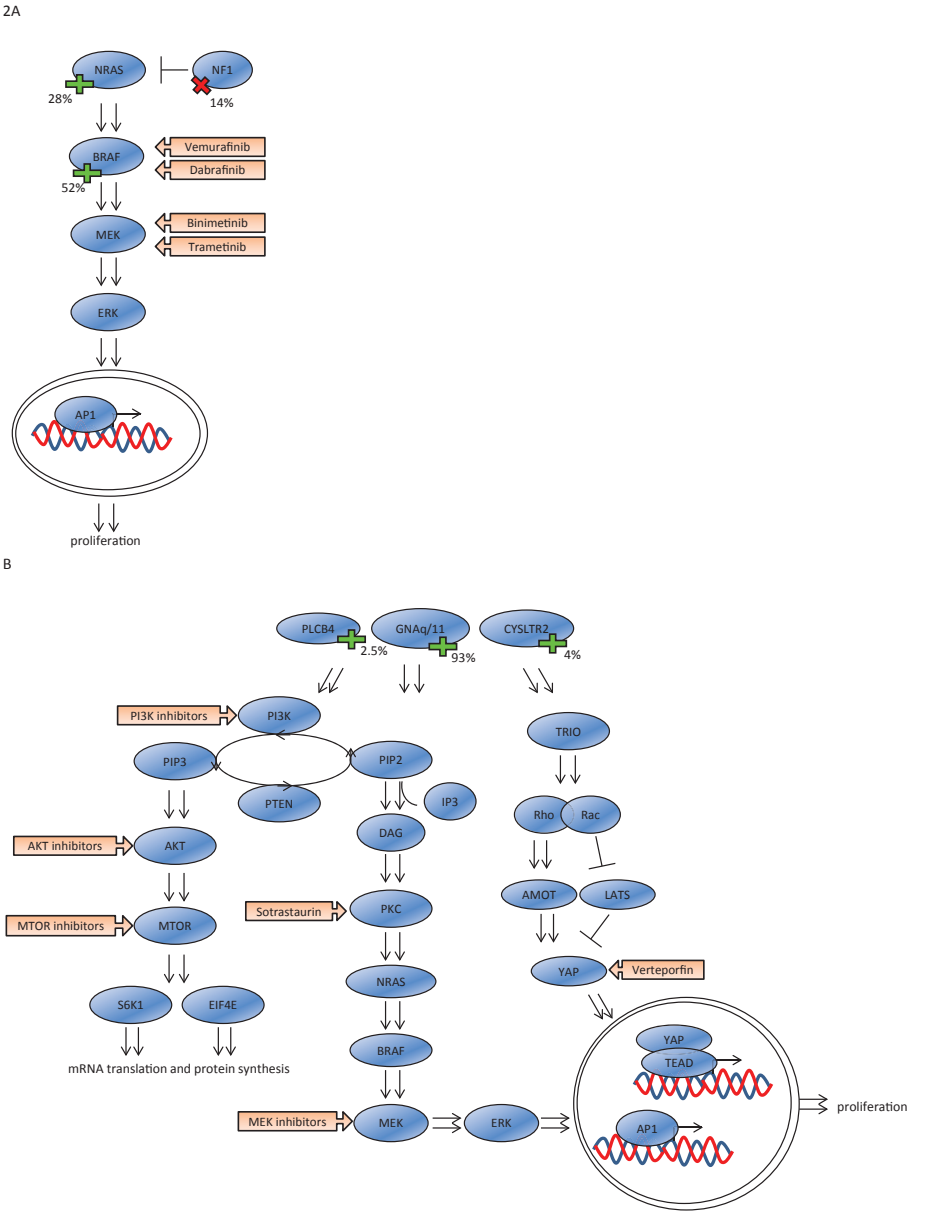

Figure 2. Melanoma signaling and therapeutic interventions. A) Cutaneous melanoma signaling driven by activating mutations in BRAF/NRAS or inactivating mutations in NF1. Therapeutic interventions consist of BRAF and MEK inhibition via Vemurafinib/Dabrafinib and Binimetinib/Trametinib respectively. B) Oncogenic mutations driving signaling in uveal melanoma. Activating mutations in PLB4, GNAQ/11 and CYSLTR2 drive the PI3K/AKT/MTOR, PKC/MEK and the YAP pathway. Therapeutic interventions in uveal melanoma therefore consist of PI3K, AKT, MTOR, PKC, MEK and YAP inhibitors

use of RTK inhibitors in these patients [106]. When patients carrying a *KIT* mutation were treated with RTK inhibitory molecules, these cancer patients develop therapy resistance by acquiring secondary NRAS mutations [107].

Knowledge about BRAF and NRAS mutations have led to the development of mutant specific BRAFV600E inhibitors and MEK inhibitors, blocking the oncogenic MAPK pathway [108]. Despite single agent success to BRAF and MEK inhibition, most patients develop disease progression after 6 to 7 months and only a small portion remain disease free [109-112]. The major factor contributing to BRAF and MEK inhibitor resistance found was the reactivation of the same MEK/ERK pathway via alternative means, such as activation of other receptor tyrosine kinases or NRAS upregulation [113-119]. MEK inhibition and NRAS depletion both trigger an apoptotic program in NRAS mutated melanoma, whereas only NRAS depletion additionally resulted in a CDK inhibitory effect. Indeed combined MEK and CDK4 inhibition resulted in synergistic therapeutic effect [120]. These results suggest that CDK4 inhibition might result in increased patient survival in combination with MEK inhibition, which is currently being investigated in an ongoing clinical trial (identifier: NCT01781572).

2.1.2 Immunotherapy

In addition to BRAF- and MEK inhibitors [109, 121, 122] the FDA has also approved immunotherapies for melanoma treatment [123, 124]. The first immune checkpoint which could be effectively targeted and inhibited was cytotoxic T-lymphocyte antigen-4 (CTLA-4) [125]. The response of a T lymphocyte, upon binding of the T cell receptor to a peptide presenting MHC molecule, is the result of a balance of both stimulatory and inhibitory signals (reviewed by [126]). This balance consists of the stimulatory interaction between CD80/86 (on the antigen presenting cell) and CD28 and the inhibitory signals residing from an interaction between CD80/86 and CTLA-4. Cancer cells take advantage of these inhibitory signals by hiding them from tumor antigen-specific T-lymphocytes. Tumor-specific antigens arise as a consequence of genomic mutations. By blocking the inhibitory signals with CTLA-4 with monoclonal antibody Ipilimumab the T-lymphocytes are unleashed and shows convincing clinical efficacy [123, 127]. Moreover, Ipilimumab was the first treatment to prolong the survival of advanced melanoma patients, highlighting the clinical importance of these therapies [123, 127].

Another effective immunotherapeutic approach is by blocking PD-1 and/or PD-1L using monoclonal antibodies. PD1 is a receptor expressed on various activated immune cells such as T-, B-, natural killer- cells and T- regulatory cells [128]. When PD1 binds to its ligand PD-1L, presented by an antigen presenting cell, the efficacy of the activated

immune cell is attenuated [129]. Like CTLA-4, PD-1/PD-1L blocking results in increased progression free- and overall survival, with a manageable toxicity profile [130-133]. Interestingly, BRAF inhibition seems to enhance PD-1/PD-1L expression suggesting that down regulating the immune system is beneficial for the acquirement of BRAF resistance [134]. These data suggested already that combining BRAF inhibition with immunotherapy could boost the efficiency of each single therapy. And indeed, pre-clinical data have shown that combining BRAF inhibition with immunotherapy has significant additive effects over the single treatments [135, 136].

2.2 Uveal melanoma

Uveal melanoma (UM) accounts for approximately 5 % of total melanoma incidence and originates from the choroid (85%), iris (5%) or ciliary body (10%) [137, 138]. Driver mutations in UM are found in the α subunits of G-proteins *GNAQ* (50%) or *GNA11* (43%), mainly resulting in a Q209L substitution locking GNAQ/11 in a GTP-bound, active state [139-141]. Due to the high frequency of these activating mutations in GNAQ/11, like BRAF in cutaneous melanoma, targeting the mutant protein(s) could potentially serve as an interesting therapeutic intervention. Although a number of cyclic depsipeptides have been reported to selectively inhibit GNAQ, it has not been investigated properly whether these compounds can still bind the mutant GNAQ [141, 142]. UM without *GNAQ* or *GNA11* have mutual exclusive mutations in the G-protein coupled receptor encoding *Cysteinyl Leukotriene Receptor 2* (*CYSLTR2)* (4%) or the downstream effector *Phospholipase C Beta 4* (*PLCB4)* (2.5%) [143, 144]. Together these data demonstrate that constitutively active G-protein signaling is an important early event in UM.

Like with cutaneous melanoma, the primary UM tumor can be treated efficiently. However, once UM patients develop metastasis, which happens in about half of the patients within 15 years after primary tumor detection, median survival is reduced to only several months since no effective treatment exists [145-147]. Frequent chromosomal aberrations in UM are loss of one copy of chromosome 3, amplification of 8q, 6p or both. Less frequently 8p gain or loss of 1p, 6q and 16q is observed [148, 149]. Monosomy 3 strongly correlates with development of metastasis and therefore is a marker for poor prognosis [150, 151]. The *BAP1* gene residing at chromosome 3 frequently shows an inactivating mutation and the remaining wild type *BAP1* allele is often lost due the monosomy 3 [152]. Mutations in BAP1 have a strong predictive power for the occurrence of metastasis in UM and 80-90% of the metastatic patients contain a *BAP1* mutation [152, 153]. BAP1 functions as a de-ubiquitination enzyme and a regulator of cell cycle progression and DNA damage response [154-157]. It is thought that BAP1 influences these processes by de-ubiquitination of one of its

primary targets, histone 2A [158]. Depletion of BAP1 *in vitro* results in a stem cell-like phenotype of UM cells [159]. In addition to monosomy 3 and loss of BAP1 expression, gain of 8q is associated with poor survival rates [160, 161]. Multiple potentially interesting genes residing on 8q could potentially explain the poor survival and/or provide interesting therapeutic targets, such as proto-oncogenes *PTP4A3*, *c-MYC*, *PVT1*, *LYN* and *MOS*.

In addition, mutations have been found in the *EIF1AX* gene, coding for Eukaryotic Translation Initiation Factor 1A X-linked, an essential component of translation initiation [162-164]. Mutations in EIF1AX occur for 20% in N-terminal end of the protein, which do not include inactivating mutations, such as frame shifts suggesting activating mutations [163, 165]. Mutations in *EIF1AX* are associated with good prognosis and subsequently correlate with disomy 3 [163]. Interestingly, only the mutant allele is expressed suggesting an oncogenic selection advantage [163]. Depletion of EIF1AX in wild type and mutant cell lines result in reduced cell viability, suggesting *EIF1AX* to be an essential gene [165]. Another gene often found mutated in UM in which two copies of chromosome 3 are retained is encoding the splicing factor 3B subunit 1 (*SF3B1)* and these mutations corrupt SF3B1 functioning and are associated with a favorable prognosis [162, 166]. However, it has recently been shown that, although *SF3B1* mutations have a favorable prognosis compared to monosomy 3 tumors, these mutations are associated with metastasis development after 5 year [167], indicating that *SF3B1* mutations are a long term poor prognosis marker. Mutations in *SF3B1* are found in 10-21% of patients and mainly affect Arg625 [163, 166]. SF3B1 has been shown to be an essential part of the spliceosome [168]. It is, therefore, not surprising that mutations in *SF3B1* resulted in alterations in the splicing of many genes [169, 170]. It remains unclear how *EIF1AX* and *SF3B1* mutations exactly contribute to melanoma formation and how their functions correlate with their respective prognostic implications. It could be hypothesized that due to the mutual exclusive pattern and functioning in RNA processing EIF1AX and SF3B1 have partly overlapping functions in driving UM.

Most novel therapeutic interventions employed for metastasized UM focus on mutated G-protein signaling. G-protein coupled signaling feeds into the know oncogenic MAPK pathway via its important effector PLC-β, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [171]. IP3, via the increase of intracellular Ca^{2++}, and DAG act as second messengers to activate various protein kinase C (PKC) isoforms (Figure 2B) [172, 173]. Although multiple PKC isoforms are activated, PKC δ and ε were shown to be sufficient to activate MEK, mediated by RAS Guanyl Releasing Protein 3 (RASGRP3) activation,

which in turn promotes UM survival and proliferation [174]. Indicating that the growth inhibitory effects of other PKC isoforms is not mediated trough MAPK inhibition. The insights into PKC activation have spurred investigations on PKC inhibitors such as Sotrastaurin. Indeed, UM cells are highly dependent on PKC and MEK signaling and were found to be sensitive to either MEK or PKC inhibition by small molecule compounds [175, 176]. A phase I clinical trial with UM patients was initiated using Sotrastaurin as PKC inhibitor. Sotrastaurin treatment resulted in progression free survival of 15 weeks in about 50% of the patients [177]. Interestingly, both MEK and PKC inhibition is required to completely abolish ERK phosphorylation and thereby cell proliferation and survival *in vitro* and *in vivo* [176]. Unfortunately, a clinical trial assessing the potency of dual MEK and PKC inhibition had to be terminated due to strong adverse effects [178]. Aside from the MAPK pathway the PI3K pathway is also activated by the continuous G-protein coupled signaling in UM (Figure 2B). Upon activation PI3K catalyzes the conversion of PIP2 into PIP3, which in turn mediates the activation of AKT [179]. Indeed, the inhibition of the PI3K/AKT pathway has been shown to reduce proliferation *in vitro* [180]. A downstream target of AKT in the PI3K pathway is MTOR, a kinase with downstream effectors 4E-BP1 and S6K1 regulating translation [181-185]. Although multiple effective MTOR inhibitors exist, the impact of mTOR inhibition on UM cell proliferation and survival appears to be far less potent when compared to BRAF mutant cells [180, 186, 187]. Mutated G-protein coupled signaling to cell proliferation and survival also involves the transcription regulators YAP and TAZ (Figure 2B). Mutated GNAQ/11 has been demonstrated to increase YAP/TAZ activity via Trio and downstream G-proteins Rho and Rac [188, 189]. The requirement of the YAP pathway for UM proliferation and survival was best illustrated by the knockdown of YAP in UM cells. Additionally, small molecule inhibition of YAP using Verteporfin demonstrated the clinical potential of targeting this pathway downstream of mutated GNAQ/11 [188-190]. Together these pathways provide a wide range of opportunities to find novel therapeutic interventions for patients with metastasized UM (Figure 2B).

3. AIM AND OUTLINE OF THIS THESIS

The focus of this thesis is uveal melanoma (UM), an ocular cancer which, once metastasized, is lethal due to lack of effective treatment options. UM is driven by an oncogenic activating mutation in the α subunit of G-proteins GNAQ or GNA11. Essentially no mutations are found in the tumor suppressor gene *p53* in UM. To represses p53 activity approximately 65% of UM tumors express high levels of the p53 inhibitory proteins MDMX or MDM2. MDMX is shown to act as p53 inhibitor by binding to its transactivation domain, rendering it inactive as a transcription factor. Interestingly, it

has been demonstrated that the oncogenic function of MDMX reaches beyond that of p53 inhibition. The aim of this thesis is to unravel the oncogenic function of MDMX and provide new treatment options for patients with metastasized UM.

Chapter 2 describes the regulation of the transcriptome by MDMX in UM. We demonstrate here that MDMX affects the transcription of genes involved in cell cycle regulation or apoptosis. This chapter also describes novel p53-independent effects of MDMX in addition to p53 inhibition, i.e. FOXO inhibition. Furthermore, a novel p53 back-up mechanism with a potential therapeutic target is proposed in this chapter.

In chapter 3 the opportunities of a combined targeting of two common signaling pathways, GNAQ/11 mutations and wildtype p53, as therapeutic intervention for metastasized UM patients is investigated. Drugs targeting these pathways, PKC- and MDM2 inhibitors, are already known to elicit strong adverse effects in patients. Genetic interference with either MDMX or PKC δ expression or activity showed that beneficial effects can already be achieved by a more specific targeting, which is presumable less toxic to the patient.

In chapter 4 it is described, opposed to what has been reported before, that enhancer of zeste homolog 2 (EZH2) inhibition poses a valuable novel therapeutic invention for UM. However, since EZH2 inhibition might take too long to exert a clinical beneficial effect, it was investigated whether EZH2 targeting would sensitize UM cells for other therapeutic strategies. Indeed, interfering with EZH2 activity synergized with HDAC inhibition, thus providing a novel treatment option for metastasized UM.

In chapter 5 it is shown that combining two clinically approved drugs, the pan-histone deacetylase (HDAC) inhibitor Quisinostat and the pan-CDK inhibitor Flavopiridol, could serve as an effective therapeutic intervention for UM patients. In addition, this combination of compounds, effectively causing apoptotic cell death in UM cells, could serve as alternative treatment option for cutaneous melanoma patients as well.

In chapter 6 the results from the preceding chapters are summarized and discussed and implications for future research and clinical implementation provided.

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