Universiteit Leiden
The Netherlands

In search of synergy: novel therapy for metastatic uveal melanoma

Glinkina, K.A.

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General Introduction

1. Uveal melanoma

1.1 Epidemiology

Uveal melanoma (UM) is a rare ocular tumor that originates from the pigmented cells located in the uveal tract [1]. Most commonly, the tumor arises from choroidal melanocytes (85-90%), but can also originate from melanocytes in the iris (3%-5%) or ciliary body (5%-8%) [2]. Incidence rates in Europe increase from South to North, from the minimum of <2 per million in registries of Spain and southern Italy up to >8 per million in Norway and Denmark [3]. These regional differences can be referred to ethnicity [4], as the risk factors include light iris color [5] and fair skin color [6]. The role of environmental UV exposure is not clear, since epidemiological analyses failed to demonstrate the correlation of UV exposure to the incidence of UM [7, 8]. Most of the patients are diagnosed after 55 years of age [3, 9], often during routine ophthalmologic examinations, as uveal melanoma is in many cases asymptomatic [10]. When the primary tumor is not too large, it can be successfully eradicated by brachytherapy [11], proton beam therapy [12] or in some cases local surgery [13]. However, up to 50% of the patients eventually develop distant metastases [14] predominantly targeting the liver (89%-93%); other common sites include lungs (24%), bone (16%) and skin (11%) (Fig. 1A) [15, 16]. According to the Collaborative Ocular Melanoma Study (COMS), in 46% patients, the liver was the only site in which metastases were detected, while 43% patients had metastases diagnosed in the liver plus other sites. This distribution was similar across primary tumor size subgroups [17]. The median survival after diagnosis of patients with hepatic metastases is approximately 4-6 months with a 1-year survival of about 10-15% [18]. Patients with metastases not involving the liver have a median survival of approximately 19-28 months with a 1-year survival of about 76% [19]. The median survival of the patients with metastatic UM hardly increased in the past decades due to lack of novel effective therapeutic options [20].

1.2 Biology of uveal melanoma

The development of effective treatment options for metastatic UM patients essentially relies on the knowledge of the tumor biology and key signaling pathways responsible for tumor proliferation [21]. Virtually all UM cases are characterized by dysregulation of the Gα-protein signaling cascade (Fig. 1B). The most recurrent alterations (90% of all cases) involve activating mutations in the genes *GNAQ* or *GNA11* encoding the α subunit of GTP-binding proteins (G-proteins) [22]. Heterotrimeric G-proteins consist of Gα, Gβ, and Gγ subunits and transduce signals from G-protein-coupled receptors (GPCR) to various intracellular signaling cascades [23]. Activated GPCRs induce the exchange of GDP for GTP on the Gα subunit and its dissociation from Gβγ. The signal is blocked when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the Gα subunit. The majority of *GNAQ/11* mutations take place within the GTPase catalytic domain, resulting in loss of the intrinsic GTPase function and constitutive activation of Gαq/11, driving cell proliferation.

Activating mutations in genes of the other members of the Gα-proteins signaling cascade, such as G-protein coupled receptor *CYSLTR2* [24, 25] or in signal mediator *PLCB4* [26], occur less often in UM. They trigger the same signaling events as *GNAQ/11* mutations, e.g. constant activation of phosphoripase PLCβ, which cleaves phosphatidylinositol bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3), and mediates activation of downstream effectors such as protein kinase C(PKC) [27] and, subsequently, the mitogen activated protein

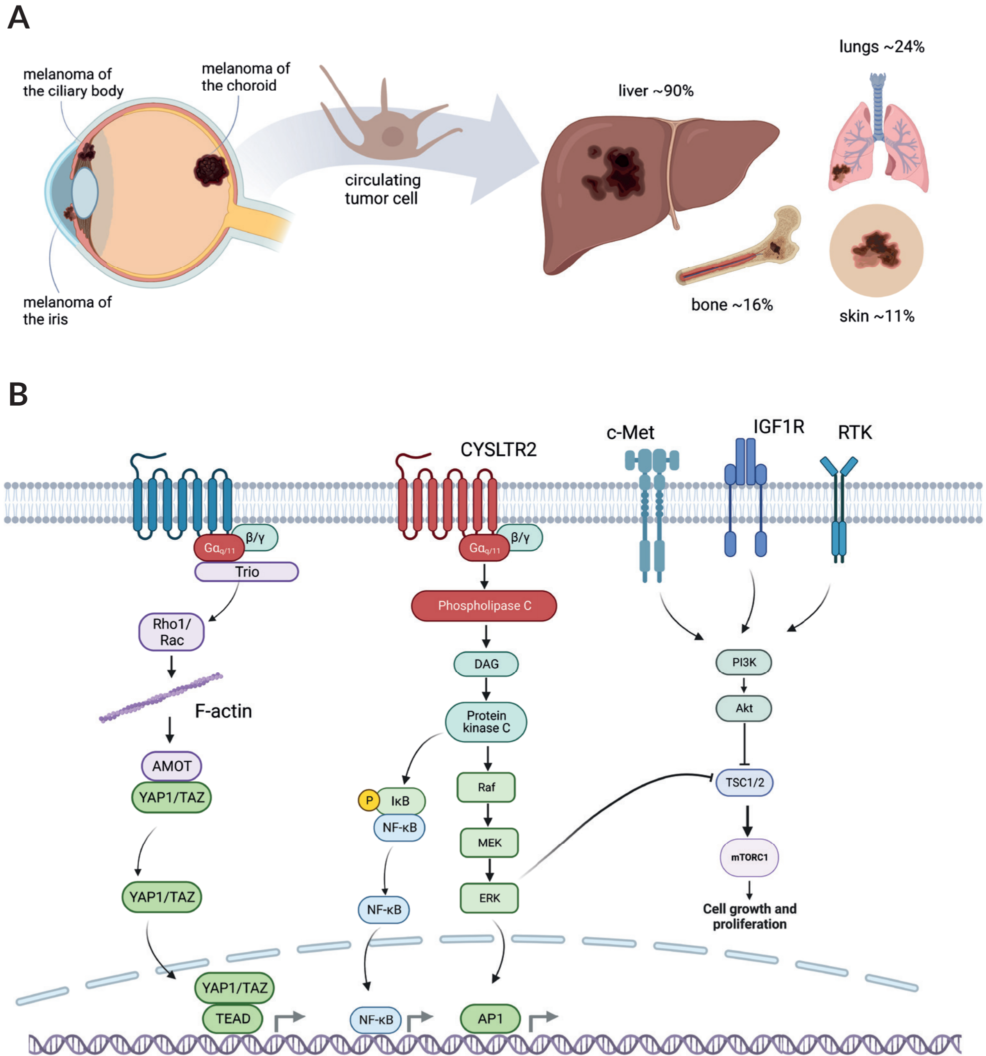

Figure 1. Uveal melanoma. (A) Schematic representation of UM metastasis to the distant organs. **(B)** Major signaling cascades dysregulated in UM. The proteins harboring driver mutations (CYSLTR2, Gαq/11, PLCβ) are marked in red.

kinase (MAPK) cascade [28] and the NF-κB [29] transcriptional program.

Independently from PLCβ, the mutated Gαq forms a stable complex with the nucleotide exchange factor Trio and activates small G-proteins RhoA and Rac1[30]. In turn, GTP-bound RhoA via several downstream effectors induces accumulation of filamentous (F)-actin, which displaces the transcription factor Yes-associated protein (YAP1) from its complex with angiomotin (AMOT) [31]. The released YAP1 may translocate to the nucleus, interact with DNA-bound co-factors and start YAP1-dependent transcription [32, 33]. YAP1 transcriptional activity was demonstrated to make a contribution to the transformation of melanocytes at the nevus state and to uncontrolled proliferation of UM [34, 35].

The prevalence of the mutations of the members of Gα-proteins signaling cascade indicates

that dysregulation of this signaling route is required to initiate tumorigenesis and occurs as an early event in evolution of UM, as also illustrated by their presence in non-malignant choroidal nevi.

Subsequently, secondary alterations may take place, namely inactivating mutations in the genes *SF3B1* (24% of all cases) and *EIF1AX* (17%), encoding a splicing modulator and a translation initiation factor respectively, and loss of expression of ubiquitin hydrolase BAP1 (45%) [36]. These mutations are nearly mutually exclusive and are associated with various grades of metastatic risk. Mutations in the *EIF1AX* gene correlate with the presence of disomy on chromosome 3 and a more favorable prognosis, while inactivation of splicing modulator *SF3B1* and, in some cases, *SRSF2* are associated with intermediate metastatic risk and late-onset metastases [37]. According to Bigot et al. [38], mutated SF3B1 changes the splicing pattern in over 1,000 junctions, thus generating immunogenic neoantigens. CD8+ T-cell clones specific for these neoantigens recognize and attack SF3B1-mutated UM cells.

Loss of chromosome 3 carrying the *BAP1* gene is a strong factor for poor prognosis. The remaining allele in UM with monosomy 3 often harbors mutations in *BAP1* gene, what leads to full abrogation of BAP1 expression and strongly correlates with metastases development. BAP1 is a ubiquitin hydrolase, regulating the ubiquitination status of histone H2A, thereby affecting the transcription of multiple genes. The role of BAP1 in tumorigenesis of UM, however, is not fully understood. According to Matatall et al. [39], depletion of BAP1 in UM cells results in down-regulation of expression of canonical genes of the melanocyte lineage (*MITF*, *TRPM1*, *TYR* and *DCT*) and gain of stem-like properties, e.g. morphological changes and up-regulation of expression of the stem cell factor *NANOG*. However, it did not result in increased proliferation, migration, invasion or tumorigenicity. More recent findings indicate a role for BAP1 in regulation of the tumor microenvironment: BAP1 loss leads to elevated expression of PROS1 (Protein S) in uveal melanocytes and melanoma cells. PROS1 is an agonist of the TAM tyrosine kinase receptors (TYRO3, AXL, MERTK), and an increase of the PROS1 level causes activation of MERTK on adjacent macrophages, causing suppressive M2-polarization [40]. Moreover, BAP1 loss in UM is associated with elevated gene expression of various cell adhesion molecules, such as cell adhesion molecule 1 (CADM1), E-cadherin (CDH1), and syndecan-2 (SDC2) which may affect tumor formation and metastasis [41].

Besides these somatic mutations, UM features copy number variation of some chromosomes. The most frequent alterations include loss on 1p, 3, 6q, 8p, 9p and gain on 1q, 6p, 8q [42, 43]. Gains on chromosome 8q were demonstrated to occur early during evolution of the primary tumor, before metastatic dissemination, and ramp-up during metastatic progression [44]. An increasing dosage of 8q commonly coincides with monosomy 3 and is associated with the greater risk of metastatic death [45]. The putative oncogenic driver located in chromosome 8 was supposed to be c-MYC, and specific amplification of the *MYC* gene was demonstrated in up to 30% of primary UM according to Parella et al. [46] However, in the TCGA database, *MYC* amplification has been detected in only 18% of cases [47]. The *PTK2* gene is also localized on 8q, encodes focal adhesion kinase (FAK), and has also been proposed to be an oncogene in UM [31]. *PTK2* mRNA is highly expressed in 54% of UM cases (TCGA), and activated phosphorylated FAK (pY397) has been found in the majority of UM cases [48]. Additionally to *MYC* and *PTK2*, Dogrusöz et al. have suggested that elevated expression of the *PRKDC* gene, as a consequence of chromosome 8q amplification, may contribute to metastatic progression in UM [49].

Receptor tyrosine kinase (RTK) c-Met is activated in a large number of UM, likely through indirect gene regulation (such as loss of negative regulator VHL) rather than through an

activating mutation or gene amplification [50]. Expression of c-Met is higher in metastatic lesions than in primary sites [51]. The ligand of c-Met, Hepatocyte Growth Factor (HGF), is highly expressed in the liver and supposedly plays a role in the dissemination of the tumor [52]. Furthermore, the HGF/c-Met axis induces activation of PI3K/AKT/mTOR cascade [53-56], which is involved in downregulation of cell adhesion molecules E-cadherin and β-catenin, stimulating motility and migration of UM cells [57]. In some cases, loss of the negative regulator PTEN might serve as an additional mechanism of AKT activation [58].

Besides c-Met and FAK, other RTKs have been found activated in UM, e.g. DDR1 [59], EGFR [60], KIT [61], and IGF1R [62], which fuel multiple downstream signals and contribute to the wide landscape of dysregulated signaling in UM.

1.3 Therapeutic options up to date

1.3.1 Targeting Gαq and Gα11

Since mutated G-protein signaling affects essentially all UM cases, it serves as a logical target for treatment. Direct targeting of Gαq/11 is possible using cyclic depsipeptides YM-254890 and FR900359[63]. Attenuation of Gαq/11 signaling in UM by these depsipeptides results in cell-cycle arrest and induction of apoptosis *in vitro* and inhibition of the xenografted tumor growth in mouse model, but does not lead to tumor regression *in vivo* [64]. Combining YM-254890 with the MEK inhibitor binimetinib further inhibits the MAPK pathway and causes tumor shrinkage in two distinct *in vivo* [65] models.

1.3.2 Targeting Protein Kinase C

Since PKC is one of the downstream effectors of the mutated Gαq/11, inhibition of PKC signaling in UM has been actively investigated. The PKC inhibitors AEB071 (sotrastaurin) and LXS196 (darovasertib) have been tested as monotreatment in phase I clinical trials for metastatic UM patients. In the AEB071 trial, 153 patients took part, of whom 4 (3%) had a partial response and 76 (50%) had stable disease. A tumor shrinkage of ≥ 10% was observed in 34 patients (22%). The median progression-free survival (PFS) was 3.5 months. 97% of patients experienced drug related adverse effects (AE). This initial trial of AEB071 demonstrated some efficacy but, at the same time, significant toxicity [66]. In the LXS196 phase I trial, 68 patients were enrolled, who received LXS196 orally either once a day (QD) or two times a day (BID). The median duration of treatment was 3.71 months and 4.6 months for patients in the QD and BID regimens, respectively. All patients in the QD regimen stopped treatment due to progressive disease. Overall, amongst 66 evaluable patients, 6 had a partial response (2 in QD; 4 in BID) and 45 had stable disease as their best response. In 25% of the patients, grade 3 or higher AEs were reported [67]. LXS196 under the name of darovasertib is currently under clinical investigation in combination with c-Met inhibitor Crizotinib. In this study by IDEAYA [68], 35 patients with metastatic UM were evaluated, and 31 (89%) patients showed tumor shrinkage; 11 (31%) evaluable patients had a confirmed partial response. All patients experienced AEs, of which 76% were evaluated as grade 1 or 2 and 24% were grade 3.

As the PKC family includes 10 different isoforms, Heijkants et al. [69] hypothesized that targeting a single PKC isoform would cause less AEs in comparison with a pan-PKC inhibitor and demonstrated that specific depletion of PKCδ inhibits UM proliferation, and this effect can be further enhanced by p53 reactivation.

1.3.3 Targeting MDM2/MDMX to activate p53

The *TP53* gene is rarely mutated in UM in contrast to many other tumor types [70], but p53's tumor suppressor function is blocked by high expression of the p53 inhibitors MDM2 and MDMX [71, 72]. Targeting MDM2 in combination with PKC or Bcl-2/w/xl inhibitors demonstrated tumor suppressing effects in xenograft (PDX) models [73, 74]. Besides MDM2, the other p53 bound protein MDMX also attenuates p53 activity and targeting MDMX might serve as an opportunity to reduce the adverse side-effects in comparison to MDM2 inhibition [69]. Furthermore, MDMX demonstrates growth-promoting functions in UM which are partially independent of p53 [72]. Recent studies demonstrated a possible role for FOXO transcription regulators in these p53-independent oncogenic functions of MDMX [75].

1.3.4 Targeting PI3K/AKT/mTOR

Transcription factors of FOXO family are substrates of the serine/threonine protein kinase AKT. Yan et al. reported that inhibition of FOXO3 by IGF-1 via the PI3K/AKT cascade is involved in IGF-1 induced proliferation and invasion of UM cells [76]. Furthermore, FOXO3 inhibits growth and survival of UM by increasing the expression of the pro-apoptotic protein BIM, and the cell cycle inhibitor CDKN1B/p27^{Kip1} [77].

The inhibitors of PI3K/AKT/mTOR axis demonstrated limited tumor suppressive activity as single agents, but could induce apoptosis in UM cell lines when combined with the other inhibitors, e.g. targeting MEK or PKC [53, 55, 56, 78, 79]. We observed a synergistic effect of the combinations of the mTOR inhibitor everolimus with either IGF1R or DNA-PKcs inhibitors on the growth of UM cell lines, but the cause of the synergism was the fast onset of cell cycle arrest rather than apoptosis [80].

Another synergistic combination, of everolimus with PI3K inhibitor GDC0941, induced apoptosis in UM cells *in vitro* and in xenograft models [81]. The synergism between the two drugs was associated with the ability of GDC0491 to block the reactivation of AKT induced by everolimus.

The clinical study of everolimus in combination with somatostatin analog pasireotide showed limited clinical benefit for a cohort of 14 metastatic UM patients, while the need for dose reductions due to the side effects was common [82].

The case study reported by Bhangoo et al. demonstrated a durable response to mTOR inhibition of a patient with a collision tumor composed of adenocarcinoma and UM metastases [83]. The disease was resistant to several lines of chemotherapy, but the treatment with temsirolimus showed clinical benefit for 6 months. The genomic profiling of patient's tumor tissue revealed loss of the *TSC1* gene, what may have resulted in hyperactivation of mTOR and sensitivity to temsirolimus.

1.3.5 Targeting the MAPK pathway

The inhibitors of MAPK pathway, e.g. MEK inhibitors, have been widely studied in the context of metastatic UM. Despite the fact that MEK inhibitors, either alone or in combination with other agents, demonstrated promising results *in vitro* and *in vivo* on a xenografted UM cell line, the outcome of clinical trials turned out to be disappointing [84-86]. The data from the clinical studies of the combinations of selumetinib with dacarbazine, trametinib with AKT inhibitor uprosertib (GSK2141795) and binimetinib with sotrastaurin, systematically reviewed

by Steeb et al. [87], demonstrated an overall response rate ranging from 0 to 14% (average 2.5%). The median progression-free survival ranged from 3.1 weeks to 16 weeks. Severe AEs were observed, mainly for the combinations of selumetinib with dacarbazine (62%) and binimetinib with sotrastaurin (75%). Recent findings pinpoint a role of histone deacetylases (HDACs) in MEK inhibitor escape in UM [88], and suggest that the combination of MEK with HDAC inhibitors might, at least temporarily, overcome the resistance to MEK inhibition [85].

1.3.6 Targeting histone deacetylases

HDACs have recently emerged as putative therapeutic targets for various tumor types. Expression of *HDAC-1*, *HDAC-3*, *HDAC-4*, as well as *HDAC-8* was found significantly higher in high-risk UMs with monosomy on chromosome 3 compared to the tumors with disomy on chromosome 3; in contrast, *HDAC-11* had a significantly lower expression in monosomy 3 tumors [89]. HDAC-2 was found the most frequently expressed isoform (66% of tumors) among class I HDAC isoforms, and the presence as well as the nuclear localization of HDAC-2 correlated with patients' improved overall survival (OS) [90].

Unlike commonly used chemotherapeutic agents that induce DNA damage both in tumor and healthy tissues, HDAC inhibitors demonstrate strong selectivity and thus exhibit less toxicity to healthy tissues. The class I HDAC inhibitor valproic acid has been shown to slow down the growth of an engrafted UM cell line *in vivo* [91]. Similar results were obtained by using the inhibitor of the class I and II HDACs Quisinostat [92]. Moreover, Quisinostat has demonstrated immunomodulatory activity on UM cell lines via upregulation of HLA class I expression [89]. Combination of Quisinostat with pan-cyclin dependent kinase (CDK) inhibitor flavopiridol synergistically enhances the tumor suppressor effect and induces apoptosis in metastatic UM cell lines [93].

1.3.7 Targeting Bcl-2 protein family

Induction of apoptosis is, among other mechanisms, regulated by the members of Bcl-2 protein family. Bcl-2 expression is elevated in primary UM compared to choroidal melanocytes and healthy ocular tissue, what is a common feature of various cancers [94-96]. Targeting Bcl-2 serves a way to overcome resistance to certain inhibitors, i.e. fotemustin, MDM2 inhibitors and the Mcl-1 inhibitor MIK665, while it demonstrates no effectivity as single treatment in UM [73, 97, 98].

Mcl-1 is a pro-survival member of Bcl-2 family, which, like Bcl-2, is expressed in UM [98]. Mukherjee et al. report sensitivity of UM cell lines to Mcl-1 inhibition, and we demonstrate in chapter 4 of this thesis that this effect can be further enhanced by blocking YAP1/TAZ-dependent transcription [97].

1.3.8 Targeting YAP1/TAZ

According to our studies (chapter 4 of this thesis), depletion of either YAP1 or TAZ expression, or pharmacological inhibition of this pathway, forces UM cells into growth arrest. We attenuated YAP1/TAZ signaling either indirectly via an inhibitor acting downstream in the mevalonate pathway, the geranyl-geranyl transferase inhibitor GGTI-298, or more specifically, by using K-975, a compound that blocks the interaction of YAP1/TAZ with the co-factors TEAD1-4[99]. Verteporfin has a similar mechanism of action as K-975, and has also been shown

to slow down UM cell proliferation and tumor formation *in vivo* [33]. Verteporfin treatment also results in lower YAP1 levels, but verteporfin has also been shown to inhibit tumor growth independently of YAP1 or TAZ expression [100, 101].

1.3.9 Immunotherapy

Besides conventional chemotherapy and targeted therapeutic agents, some immunotherapeutic agents have been investigated as putative treatment for patients with UM metastases.

An important general tumor-escape mechanism and a characteristic of tumor progression is represented by a downregulation of the expression of human leukocyte antigens (HLAs). In most tumor types, the HLA Class I expression is decreased on metastases compared to primary tumors. Tumor cells that have lost HLA Class I antigens on their surface evade lysis by specific T cells, but may become more prone to recognition by Natural Killer cells. Interestingly, opposed to the commonly accepted paradigm, lack of HLA Class I expression on UM cells correlates with better survival of the patients, and HLA Class I is often highly expressed in metastases of UM [102].

Tebentafusp is a bi-specific protein able redirect specific T cells towards UM cells by simultaneous binding to CD3 on the T-cell membrane and to the gp100-HLA-A*02:01 complex on UM cells. A phase 3 trial on HLA-A*02:01-positive metastatic UM patients demonstrated encouraging outcome. Overall survival at 1 year was 73% in the tebentafusp group and 59% in the control group, while progressionfree survival also significantly increased in the tebentafusp group compared to the control group (31% vs. 19% at 6 months). Most common AEs were skin-related or cytokine-mediated and included pyrexia, pruritus, and rash. Generally, these AEs became less frequent and severe after the first 3-4 doses and were managed by standard interventions [103].

Immunotherapeutic targeting of CTLA-4 or PD-1 has been accepted as a promising option for metastatic cutaneous melanoma but has failed to improve OS of the patients with metastatic UM. A number of trials of anti-CTLA-4 antibody ipilimumab at a concentration of 3 mg/kg showed no clinical benefit [104-106]; the dose increase up to 10 mg/kg led to a prolonged median OS of up to 9.8 months. However, the overall response remained low and patients experienced numerous AEs [107].

The efficiency of PD-1 blocking antibody nivolumab was assessed in the CheckMate 172 trial [108]. A subgroup of 34 UM patients resistant toanti-CTLA-4 therapy was evaluated. Two partial responses were achieved (overall response rate 6%), and 15 patients (44%) had stable disease after a follow-up of 1 year. The median OS was estimated 11 months, what is longer than the median OS for ipilimumab in this patient group.

Currently, the tumor mutational burden is recognized as a biomarker to predict sensitivity to immunotherapy. In contrast to cutaneous melanoma, UM carries remarkably low mutational burden of around 0.5 mutations per Mb sequence [26]. Therefore, neoantigens that are recognizable for T cells is unlikely to appear. Moreover, PD-L1 expression was detected in only about 10% of UM primary tumors and 5% of the cells in metastatic UM sites [109, 110]. Interestingly, Rodrigues et al. [111]. have recently presented a case of a metastatic UM patient with an exceptional response to a PD-1 inhibitor associated with *MBD4* germline deleterious mutation and somatic MBD4 inactivation by loss of the second allele of chromosome 3 in the tumor. MBD4, being part of the base excision repair machinery, recognizes and removes uracil from a G:U mispair and thymine from a G:T mispair, and, additionally, excises thymine glycol

(Tg) from a Tg:G mispair [112, 113]. Loss of MBD4 activity, therefore, results in a hypermutated phenotype and predisposes to certain types of cancer, in particularly UM [114]. The observed MBD4-related high mutation load may explain the patient's dramatic response to immune checkpoint inhibitors [115].

2. Screening strategies for drug discovery

2.1 Compound screening

Discovery of an effective small-molecule compound is a challenging multidimensional problem. Screening, in the context of drug discovery, is a tool to search for interaction of a chemical compound (either natural or synthesized) with a target. Screening aims to select members of a chemical library that interact with a biological system in a defined way [116].

Recent advances in technologies of liquid handling, imaging and software provide a basis for greater automation of different steps of the screening process. This accelerates the time for compound discovery and optimization and enables high throughput, reaching 100.000 tested compounds per day [117]. High throughput screens have been applied in a great variety of research areas and resulted in discovery of multiple chemotherapeutics.

Plenty of novel chemotherapeutics are being discovered and enter clinical testing, however, due to the limited therapeutic window of the majority of drugs, the toxicity of antineoplastic drugs remains one of the major reasons for patient drop out and discontinuation of trials [118]. The issue of excessive toxicity could be solved by increasing drug selectivity, either by modifying the compound composition, or, alternatively, by using a synergistic combination of two drugs [119, 120]. The synergistic combination of drugs might allow countering the mechanisms of biological compensation, thus increasing therapy efficiency, reducing the dosage of individual drugs and potential side-effects. One of the concepts explaining the mechanism of drug synergism might be "synthetic lethality". Two genes are synthetically lethal if mutations of both genes lead to cell death while a mutation of either alone is compatible with viability [120]. Therefore, targeting a gene (encoded protein) that is synthetically lethal to a cancer relevant mutation should selectively affect the viability of cancer cells. Similarly, two drugs targeting cancer relevant mutations or a mutation and a resistance mechanism may result in a synergistic effect. This concept is getting more widely exploited as a result of the increased availability of chemical and genetic tools for perturbing gene function in cells.

The human genome consists of a great variety of genes (>25,000) and an even larger number of gene variants and proteins (>100,000), but the number of molecular targets with approved drugs targeting these targets is still limited (~1000) [121, 122]. Some targets might not be suitable for modulation by small molecules; others might not be approachable by current technologies. Genetic screening serves a approach to widen the putative targets for search and provides a tool for identifying genes and pathways determining a phenotype or biological mechanisms.

2.2 Genetic screening

2.2.1 RNAi

RNA interference (RNAi) is an endogenous cellular process, conserved in most eukaryotic species. RNAi mechanism includes cleavage and degradation of the transcript after

recognition by a sequencespecific small interfering RNA (siRNA). siRNAs are ~21–22 bp long, double stranded RNA (dsRNA) molecules that have characteristic 3'- overhangs that allow them to be recognized by the RNA-induced silencing complex (RISC) [123]. Within RISC, the double-stranded siRNA is processed into a singlestranded "guide" RNA molecule that complementary binds to its target sequence and targets it for subsequent cleavage by endonuclease Ago2 [124].

First identified in the small nematode, *C. elegans*, the RNAi process could also be applied to mammalian cells in order to knock down the expression of genes of interest [125]. In mammals application of dsRNAs to trigger RNAi was initially hindered due to activation the interferon response by these molecules [126]. Currently, the most common approaches utilize either synthetic siRNAs, designed to mimic endogenous 21-nt siRNAs with 2-base overhangs at both 3'- ends, or short hairpin RNAs (shRNAs) expressed within the cell by introduced vector-mediated production [127, 128]. siRNA and shRNA use the same cellular mechanism and the choice between them depends on experimental parameters such as cell type and transient expression versus stable integration.

Initially RNAi technology was used to knock down the function of individual genes, but eventually production of large-scale RNAi libraries allowed genome-wide loss-of-function screenings [129].

These genome-wide screenings may be performed in arrayed or pooled format. In the arrayed assay, siRNA targeting a particular gene placed in an individual well in a 96- or 384-well plate. Detection is typically done by measuring fluorescent or luminescent readouts with a plate-reader or using microscopy. Arrayed screens have an advantage of detection of multiple phenotypes in a single experiment.

A number of assay plates must be evaluated to reach genome scale with an arrayed screen, what makes the experiment laborious and costly. The pooled screen format may overcome these drawbacks. In a pooled screen, the RNAi library (for mammalian cells, typically shRNA library), containing a pool of sequences targeting a wide subset of genes, is introduced into cells in such a way that an individual cell will carry one specific RNAi sequence. Then a screen is performed, selecting the cells resistant to a defined treatment survive. RNAi reagents present in surviving cells are identified by sequencing. The enrichment of a particular RNAi sequence after selection suggests that knockdown of the corresponding gene assigns resistance to the treatment. For practical reasons, pooled screens are primarily applied for discovery, while arrayed screens are often used for validation and follow-up investigation.

RNAi screening in mammalian cells has made possible identification of putative oncogenes and novel therapeutic targets [130-134].

RNAi silencing machinery is present in virtually every mammalian somatic cell. Therefore, no introduction of expression vector is required, and a simple siRNA transfection can cause a loss-of function phenotype.

RNAi technology still has several limitations. First, the efficiency of knockdown of the targeted gene may vary widely, making it very difficult to predict the efficiency of the silencing without experimental test [135]. Second, siRNA might bind off target mRNA sequences targeting them for degradation, thus it might be difficult to separate genuine hits of the screen from false positives [136]. Nowadays, the RNAi screening technology is being outperformed and replaced by CRISPR-Cas9 screens.

2.2.2 CRISPR-Cas9

CRISPR screens take advantage of the flexibility and efficiency of CRISPR-Cas9 genome editing [137]. The Cas9 DNA nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system is directed by small guiding RNAs (gRNAs) through complementary base pairing to a target sequence on genomic DNA, where Cas9 generates double strand breaks (DSBs). Cleavage by Cas9 triggers one of the major pathways for DNA damage repair: either the error-prone nonhomologous end joining (NHEJ) or the high-fidelity homology-directed repair (HDR). In the absence of a repair template, DSBs are re-ligated through the NHEJ process, which leaves insertion/deletion (indel) mutations. Indels that occur within the exons can cause frameshifts or formation of a premature stop codon, resulting in gene knockout.

CRISPR-Cas9 represents a system that is specific, easy to use, efficient and well-suited for highthroughput application in various cells and organisms. Similarly to RNAi screens, CRISPR-Cas9 screens can be arrayed or pooled.

In a typical pooled CRISPR-Cas9 screen, a library of guide RNAs is introduced in bulk into cells with a low multiplicity of infection, so that an individual cell receives only one particular gRNA. The gRNAs are commonly inserted in a lentiviral vector and are integrated into the genome target cells, making it possible to define the induced perturbations by the gRNA sequence. Cas9 protein can be either stably expressed in the cells or introduced by plasmid transfection, virus-mediated (e.g. adenovirus), mRNA- or protein-transfection. Then the gene-edited cells are put under selective pressure such as cell proliferation, drug treatment or viral infection. The gRNAs are then identified in the pool of the surviving cells by high-throughput sequencing and the gRNAs repertoire composition is compared between different conditions or time points.

Unlike RNAi that target mRNA for degradation after transcription, CRISPR-Cas9 induces indels in genomic DNA, what allows more efficient silencing of the target gene. A typical pooled library contains 2 to 10 distinct gRNAs targeting a specific gene. The gene editing efficiency of these gRNAs may vary, causing screening noise, which still makes it important to verify the resulting hits. The number of offtarget effects of CRISPR-Cas9 is supposed to be significantly reduced comparing to RNAi [138]. Currently CRISPR-Cas9 screening technology is gaining popularity and is extensively utilized for various research questions.

3. Aim and outline of this thesis

Metastatic uveal melanoma is an aggressive tumor resistant to commonly used anti-neoplastic therapeutics. Since the median overall survival after diagnosis of patients with hepatic metastases does not reach a year, and the median survival of patients with metastases not involving the liver is very limited as well, novel effective therapeutic options are required. In this thesis we focus on a search for novel approaches to treatment of metastatic uveal melanoma. We exploit genetic screening techniques to identify new targets and performed compound screens to identify combinations of inhibitors that act synergistically to interfere with the growth of UM cells.

In Chapter 2 we demonstrate that combinations of the multitarget drug Trabectedin with either the CK2/Clk double-inhibitor Silmitasertib or with the c-MET/TAM receptor inhibitors Foretinib and Cabozantinib show synergistic growth inhibitory effects and induce apoptosis of UM cells *in vitro*. In case of Foretinib and Cabozantinib, attenuation of activity of the TAM

receptors, particularly MERTK, but not of c-Met, is essential to inhibit proliferation of UM cells. Trabectedin alone or in combination with Cabozantinib inhibits tumor growth in PDX UM mouse models.

Chapter 3 describes the application of a CRISPR-Cas9 synthetic lethality screen for identification of molecular targets whose inhibition synergistically enhances the effect of the mTOR inhibitor everolimus in UM cells. *IGF1R* and *PRKDC* among other genes were identified as hits in the screen. The combinations of the IGF1R or DNA-PKcs inhibitors with everolimus synergistically slow down cell proliferation but do not induce apoptosis in UM cell lines. These combinations have been evaluated on PDX UM in an *in vivo* model, but did not demonstrate tumor regression. However, we could find significant activity of the dual DNA-PKcs/mTOR inhibitor CC-115 on PDX UM in an *in vivo* model.

In Chapter 4 we show that the combination of genetic depletion YAP1/TAZ together with Mcl-1 inhibition resulted in a synergistic inhibitory effect on the viability of UM cell lines. Similarly, indirect attenuation of YAP1/TAZ signaling pathway with an inhibitor of the mevalonate pathway, i.e. the geranyl-geranyl transferase inhibitor GGTI-298, synergized with Mcl-1 inhibition to antagonize UM cell proliferation.

In Chapter 5 we analyzed the phospho-proteome of two UM metastatic cell lines and a primary tumor cell line from the same individual, and studied the role of MARK3 in UM progression.

In Chapter 6 we summarize and discuss our findings in the context of existing knowledge.

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