Novel functions of MDMX and innovative therapeutic strategies for melanoma

Heijkants, R.C.

CHAPTER 4

Combined EZH2 and HDAC inhibition as novel therapeutic intervention for metastasized uveal melanoma

R. C. Heijkants, A. F. A. S. Teunisse, A. G. Jochemsen

Department of Cell and Chemical Biology, Leiden University Medical Centre, Leiden, the Netherlands.

Summary

Purpose: Currently there is no effective therapeutic intervention available for patients with metastasized uveal melanoma (UM) resulting in poor prognosis. Loss of the tumor suppressor BAP1 is frequently found (80-90%) in metastasized UM. Expression of enhancer of zeste homolog 2 (EZH2), a methyltransferase and an essential component of the polycomb repressive complex 2 (PRC2), is frequently dysregulated in cancer. Like UM, mesotheliomas frequently lack BAP1 expression and it was found that loss of BAP1 expression sensitize these cells for EZH2 inhibition. However, UM cell proliferation was reported not to be affected by EZH2 inhibition, independent of BAP1 expression. Here we continued studying the potential of EZH2 inhibition as therapeutic strategy for metastasised UM.

Methods: A panel of UM cell lines was used to determine the effects of EZH2 inhibition on both short and long term proliferation assays. Using the same cell lines the combination of EZH2 and histone deacetylase (HDAC) inhibition was assessed on cell proliferation, western blotting and flow cytometry.

Results: Here we demonstrate that UM cells are responsive to EZH2 inhibition in a long term growth assay. Furthermore, EZH2 inhibition sensitized UM cells for histone deacetylase inhibition even in a short term growth assay, correlating with increased induction of cell death.

Conclusions: EZH2 inhibition, opposed to what has been suggested previously, could still serve as a potential therapeutic intervention for metastasized UM when combined with other treatments opening new avenues for the treatment of metastasized UM patients.

Introduction

Uveal melanoma (UM) is an ocular malignancy originating from melanocytes located in the choroid (85%), iris (5%) or ciliary body (10%) [1, 2]. Primary tumors can usually be treated efficiently, however, approximately half of the patients within 15 years after primary tumor detection will develop metastases, for which no effective treatment exists to date [3, 4]. In addition to the driver mutations in the G-proteins GNAQ or GNA11, monosomy 3 and amplification of 8q are frequently observed genomic aberrations in UM [5, 6]. Particularly monosomy 3 strongly correlates with development of metastases and is, therefore, a robust marker for poor prognosis [7, 8]. The *BAP1* gene is located at chromosome 3 and in monosomy 3 tumors the remaining copy of *BAP1* is often found mutated leading to complete loss of BAP1 protein expression [9]. Indeed, mutations in *BAP1* have a strong predictive power for the occurrence of metastasis in UM and 80-90% of the UM metastases show loss of *BAP1* expression [9, 10]. Interestingly, a previous study reported upregulation of e*nhancer of zeste (EZH) 2* expression in mesothelioma upon *BAP1* loss [11].

EZH2 is frequently overexpressed and also mutated in various cancer types, including melanoma, and its high expression correlates with disease progression and aggression (Reviewed by [12]). EZH2 is an essential component of the polycomb repressive complex 2 (PRC2) and functions as a methyltransferase, catalysing the tri-methylation of histone H3 at lysine 27 (H3K27me3) [13, 14]. This repressive tri-methylation mark of histone H3 is recognized by the PRC1 complex resulting in gene silencing [15, 16]. In this way EZH2 controls the transcription of numerous genes [17]. In addition to the transcription repressive function of EZH2 in the PRC2 complex, it has been demonstrated that EZH2 is capable of promoting transcription, independently of PRC2 complex [18]. The switch from transcription repressor to activator appears to be mediated by the phosphorylation of serine 21. All in all, EZH2 emerges as an important regulator of transcription in cancer cells.

Transformation of *BAP1* knockout myeloid cells was found to be *EZH2*-dependent and EZH2 inhibition was demonstrated to be an effective treatment for BAP1-negative mesothelioma in a pre-clinical *in vivo* model [11]. Based upon these results a clinical trial with the EZH2-inhibitor Tazemetostat on patients with BAP1-negative malignant mesothelioma is ongoing (NCT02860286). These observations in malignant mesothelioma could be extrapolated to *BAP1*-negative metastasized UM, possibly providing an effective therapeutic intervention. However, a follow-up study addressing this matter reported that UM cell lines are insensitive to EZH2 inhibition regardless of *BAP1* expression, disputing the generality of the observations made in malignant

mesothelioma [19]. However, it was argued that UM cell lines might need a prolonged exposure to EZH2 inhibition to observe growth inhibitory effects, especially because *BAP1*-negative UM cell lines generally have a very long doubling time [20]. This study focuses on the long term effects of EZH2 inhibition on UM cells and addresses the combinatory use of EZH2 inhibitor with histone deacetylase (HDAC) inhibition as potential therapeutic strategy for metastasized UM.

Results

EZH2 inhibition slows down uveal melanoma cell growth.

First, the biochemical effect of EZH2 inhibition, namely the reduction of H3K27me3, was confirmed at different time points with all distinct EZH2 inhibitors tested in the current study (T: Tazemetostat, E: EPZ011989, G: GSK503 and U: UNC1999) compared to DMSO (D) treated cells (Fig. A). The reduction of H3K27me3 after 24 hours incubation with EZH2 inhibitors is modest, but strong effects could be observed after 48, 72 and 96 hours. Although it must be noted that UNC1999 appears less potent since this drug is not able to completely abolish detectable H3K27me3 in time, in contrast to the other EZH2 inhibitors (Fig. 1A). To assess the long term effects of EZH2 inhibition on growth of UM cells, cells were seeded into 6-well plates and cultured with or without exposure to two distinct EZH2 inhibitors (EPZ011989 or Tazemetostat), each condition in duplicate. When control (DMSO)-treated cells reached 80-90% confluency the cells from all conditions were counted to determine the effect of EZH2 inhibition on the growth, and all conditions were re-seeded in the same density as before to ensure equal conditions during this long term assays. Only minimal or no growth inhibition was observed within 5-9 days of treatment matching previous reported data of EZH2 inhibition in UM cells (Fig. 1B) [19]. Results of all cell counts show different dynamics per cell line upon EZH2 inhibition (Supplementary Fig. 1A). Interestingly, the BAP1-positive cells (OMM1, OMM2.5 and MM66) could still be sub-cultured with continuous EZH2 inhibition even though EZH2 inhibition resulted in clear growth retardation of OMM1 and MM66 cells. OMM2.5 was the only cell line tested whose growth was hardly affected by EZH2 inhibition, even after 40 days (Supplementary Fig. 1A).

Interestingly, two out of three BAP1-negative cells (MP38, MM28) completely stopped proliferating after 1 or 2 passages (Supplementary Fig. 1A). These data slightly hint towards a higher efficacy of EZH2 inhibition in BAP1-negative cells, although more BAP1-negative and positive cell lines need to be tested to confirm this result. However, supporting this possibility is that analysis of TCGA data indicates that *EZH2* mRNA expression is significantly upregulated in UM samples with a deleted *BAP1* allele

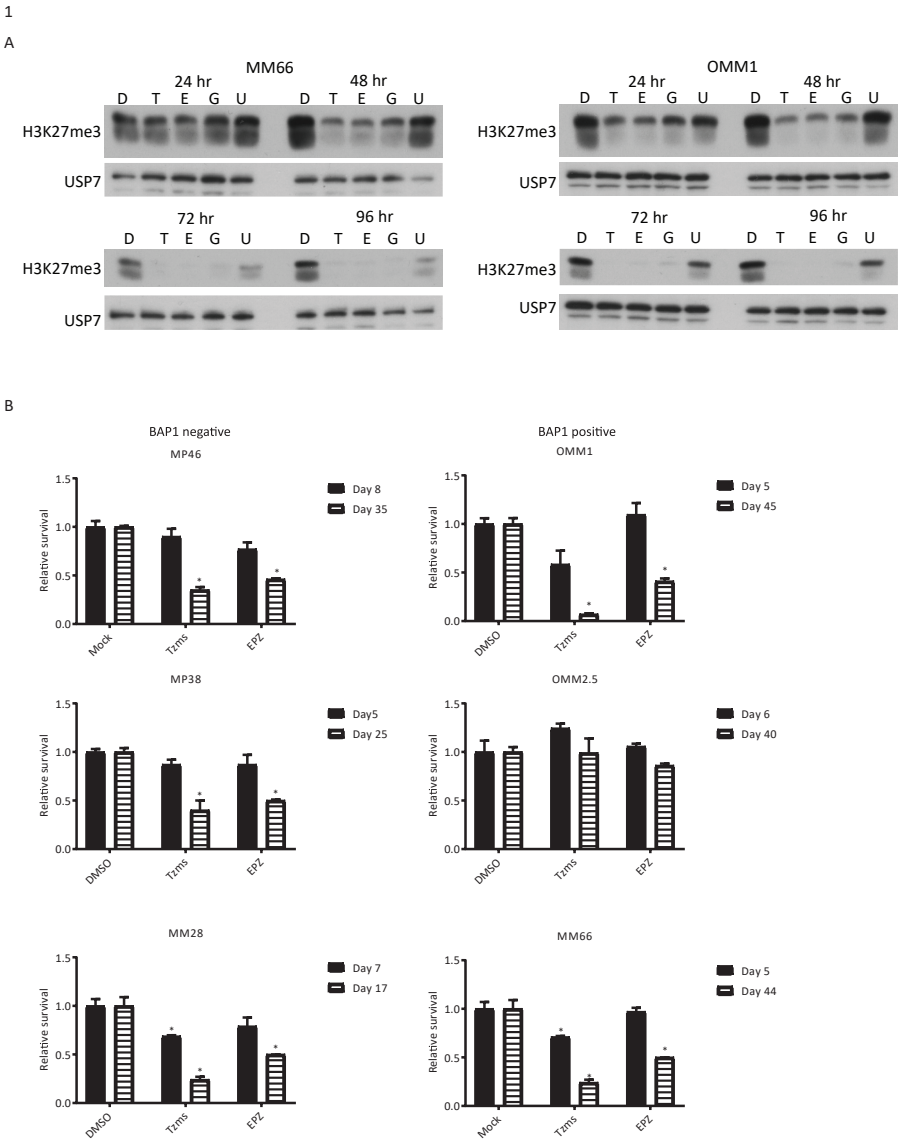
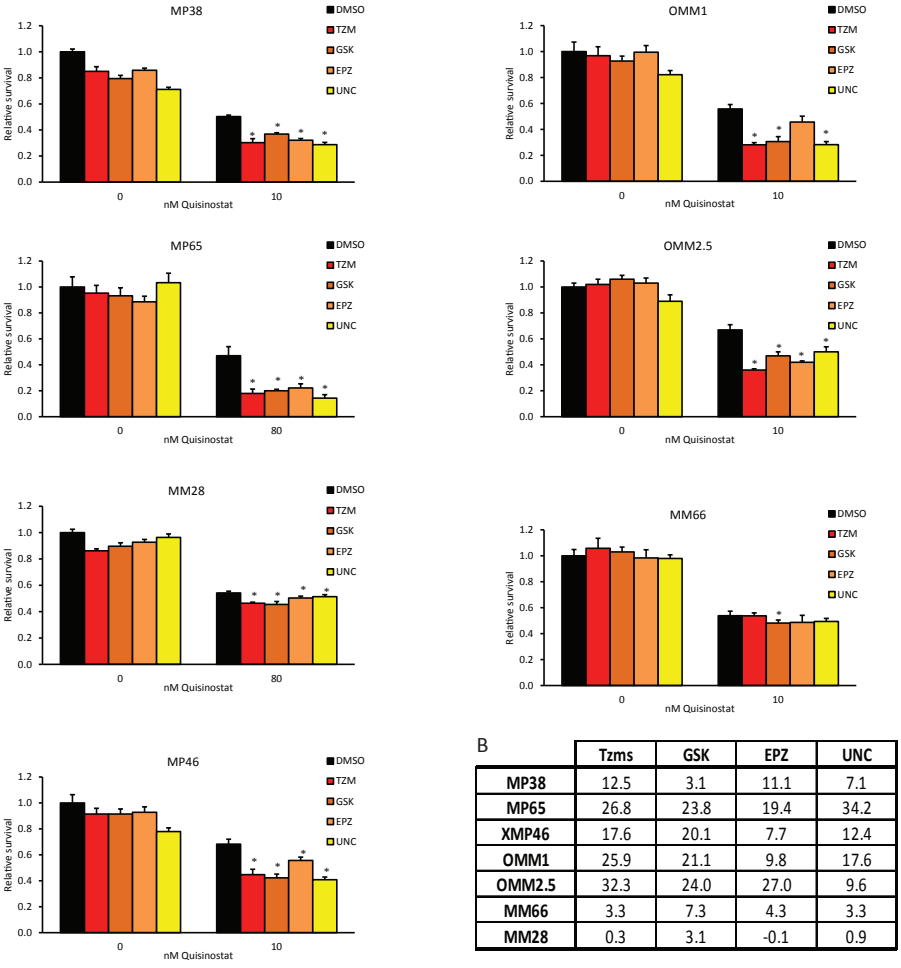


Figure 1. Effects of EZH2 inhibition on long-term growth of BAP1-positive and -negative uveal melanoma cell lines. (**A**) Time-course of four distinct EZH2 inhibitors (Tazemetostat (T; 6 µM), EZP011989 (E; 6 µM), GSK503 (G; 6 µM), UNC1999 (U; 4 µM) and control (DMSO-treated) to investigate kinetics of H3K27me3 reduction; USP7 expression is analysed to show equal loading. (**B**) Early and last data points of proliferation assay of BAP1-positive (OMM1, OMM2.5 and MM66) and BAP1-negative (MM28, MP38 and MP46) uveal melanoma (UM) cells cultured in duplicate in the continuous presence of EZH2 inhibitors (2 µM Tzms (Tazemetostat) or 3 µM EPZ (EZP011989)). When DMSO treated cells reached 80-90% confluency the cells from all wells/conditions were trypsinized and counted and re-seeded with the same initial density to continue the assay.

2
A



B

	Tzms	GSK	EPZ	UNC
MP38	12.5	3.1	11.1	7.1
MP65	26.8	23.8	19.4	34.2
XMP46	17.6	20.1	7.7	12.4
OMM1	25.9	21.1	9.8	17.6
OMM2.5	32.3	24.0	27.0	9.6
MM66	3.3	7.3	4.3	3.3
MM28	0.3	3.1	-0.1	0.9

Figure 2. Synergistic effects of combined EZH2- and HDAC inhibition on uveal melanoma. (**A**) Indicated cell lines were treated with 4 distinct EZH2 inhibitors, Tazemetostat (Tzms; 4 µM), GSK (GSK503; 4 µM), EZP011989 (EPZ; 4 µM for MM28, MP65 and MM66; 6 µM for MP38, MP46, OMM1 and OMM2.5), UNC1999 (UNC; 1 µM for MM28, MP65, OMM1, OMM2.5 and MM66; 4 µM for MP38 and MP46) or DMSO, the HDAC inhibitor Quisinostat or by a combination. Asterisk (*) indicates significant differences ($p \leq 0.05$) between Quisinostat-treated and the combined treated cells. (**B**) Excess over Bliss values were calculated to determine synergism.[39]

(Supplementary Fig. 1B) [21]. No such upregulation was found for SUZ12 expression, another member of the PRC2 complex, showing that the EZH2 upregulation was

not due to a general increase of all members of PRC2. In accordance with previous observations for mesothelioma cells [11], our results are supporting the argument that BAP1-negative UM cells are more sensitive to EZH2 inhibition, although the differences are clearly less dramatic in UM compared to mesothelioma.

Synergistic effects of concurrent HDAC and EZH2 inhibition.

Due to the lack of a rapid onset of growth arrest, EZH2 inhibition most likely will not be effective as single treatment for metastatic UM patients. It has previously been shown that dual inhibition of EZH2 and HDACs strongly reduced tumor cell survival and, therefore, has an interesting therapeutic potential [22-24]. For this reason we tested the broad spectrum HDAC inhibitor Quisinostat (JNJ-26481585), with known pre- and clinical effects in (uveal) melanoma cells and patients [25-27], in combination with four distinct EZH2 inhibitors. As mentioned before, EZH2 inhibition did not or hardly affect UM survival after an incubation of 5 days (Fig. 2A). Even so, all EZH2 inhibitors clearly enhanced the growth inhibition by Quisinostat in a synergistic manner in most cell lines (Excess over Bliss values $\geq$2) (Fig. 2A and B).

Combinatory inhibition of HDAC and EZH2 induces uveal melanoma cell death.

Analysis of protein lysates of tested cell lines showed a marked increase in H3K9/14-acetylation and a decrease of K27 tri-methylation upon Quisinostat or Tazemetostat, respectively (Fig. 3A). These changes in epigenetic markers show that both compounds efficiently affect activity of their designated target proteins. In most cell lines the combination stimulated cell death by apoptosis as indicated by increased PARP cleavage (Fig. 3A). Only MM66 did not show an increase in PARP cleavage in the combination compared to single Quisinostat treatment. MM28 hardly showed any PARP cleavage, suggesting resistance to apoptosis induction (Fig. 3A). Combination of Quisinostat with GSK503 yielded similar results in four cell lines tested (data not shown). Surprisingly, in both MM28 and MM66 the changes in epigenetic markers clearly indicate EZH2 and HDAC inhibition upon drug treatment, rendering the lack of synergism not due to inefficient target inhibition but to not yet identified differences between these cell lines. To verify increased cell death by the combined treatment, flow cytometry analyses have been performed. As expected, both EZH2 inhibitors Tazemetostat and GSK503 hardly affected the cell cycle profile correlating with the lack of effect on cell proliferation (Fig. 3B). Quisinostat on the other hand elicited a clear G1 phase cell cycle arrest, in accordance with our earlier and previous studies with other HDAC inhibitors [27, 28]. Importantly, combined treatment increased the fraction of subG1 cells in most cell lines (Fig. 3B), in agreement with the PARP cleavage analyses. As with the previous experiments hardly any differences were found between the single treat-

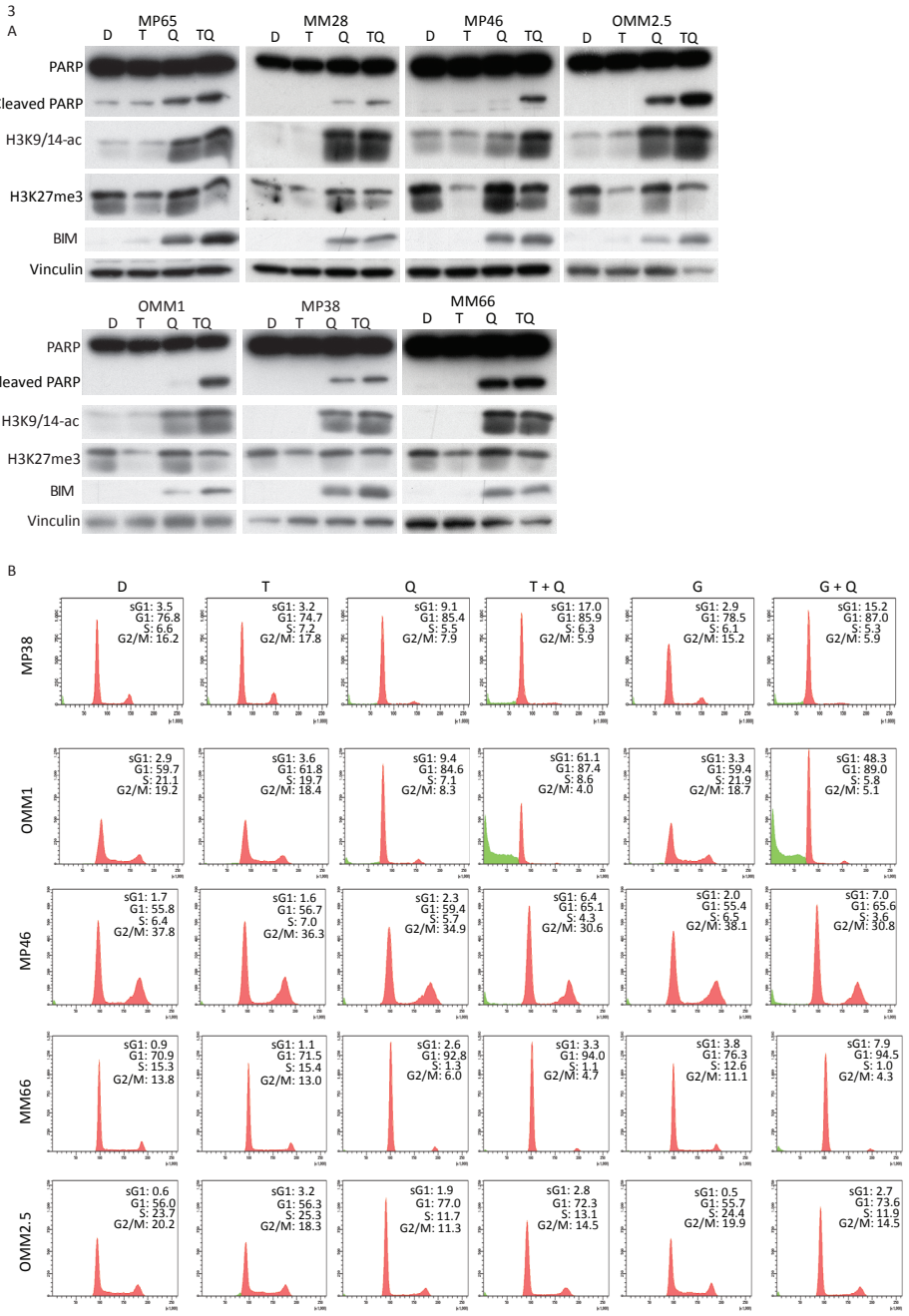


Figure 3. Induction of cell death upon concurrent inhibition of EZH2 and HDAC. (**A**) Biochemical effects of EZH2 and HDAC inhibition on BAP1-positive and -negative UM cells were assessed after 48 hours by Western blot. Tazemetostat (T; 4 μM) reduced tri-methylated lysine 27 of histone 3 (H3K27me3) and Quisinostat (Q; 40 nM) treatment enhanced histone 3 acetylation (H3K9/14-

ac). Pro-apoptotic BIM protein levels and PARP cleavage were analysed as apoptotic markers and expression of vinculin was analysed to show equal loading. (**B**) Effects of EZH2 inhibition (T; Tazemetostat; 4 μM) and G; GSK503; 4 μM), HDAC inhibition (Q; Quisinostat; 40 nM) or combinations on the cell cycle profiles of UM cells after 48 hours.

ments and the combined-treated MM66 cells although a slightly increased subG1 phase is found in the Quisinostat/GSK503 combination.

To find an explanation for the increased cell death upon combinatory treatment expression of a number of genes involved in apoptosis was investigated, i.e. *BIM*, *NOXA* and *Survivin*.

These genes were chosen because it had been shown that *BIM* expression is strongly increased by combined HDAC and EZH2 inhibition [29] and both *NOXA* and *Survivin* have been reported to be responsive to HDAC inhibition [30, 31]. Indeed, Quisinostat is strongly increasing the mRNA levels of *BIM* and *NOXA* and in combination with EZH2-inhibition by Tazemetostat the levels are further enhanced, although this increase is not always statistically significant (Supplementary Fig 2). BIM protein levels were also investigated and indeed in most cell lines the combined Quisinostat/Tazemetostat treatment resulted in further increase in BIM levels (Figure 3A). As reported, HDAC inhibition strongly downregulates the expression of *Survivin*, which is not affected by addition of Tazemetostat. Since several reports that HDAC inhibition increases levels of FOXO proteins and the *BIM* gene is a known FOXO target, we investigated the expression of *FOXO1, FOXO3 and FOXO4* mRNA. Also in these tested UM cell lines HDAC inhibition increased *FOXO1* and *FOXO3* mRNA levels and in 2/3 cell lines the increase is enhanced by concomitant EZH2 inhibition (Supplementary Fig 2). *FOXO4* mRNA levels were also increased upon HDAC inhibition in all UM cell lines tested, although less prominent, and was not further enhanced when HDAC and EZH2 inhibition was combined.

Thus, EZH2 inhibitors do not affect UM cell proliferation or survival at early time points but they still might sensitize UM cells for other therapeutic interventions, as illustrated here by HDAC inhibition. These effects are likely to be mediated in part by the upregulation of FOXO transcription factors.

Discussion

Despite ongoing developments with regard to novel therapeutic strategies to treat metastasized UM, no effective curative treatment is available. Typical for the onset of metastasis in UM is the loss of one chromosome 3 and inactivating mutations in the remaining *BAP1* gene [10, 32]. These events result in the absence of BAP1 expression in 80-90% of the metastasized UM cases [10]. The observation that cells lacking BAP1 expression would be more sensitive for EZH2 inhibition would, therefore, meet the need for a specific treatment of UM metastases. The data presented in this study suggest that UM cells are sensitive for EZH2 inhibition upon long-term treatment, seemingly in contrast to the conclusions drawn by Schoumacher *et al.*[19]. Same cell lines and partly the same compounds (Tazemetostat = EPZ6438) were used, but the apparent discrepancies can easily be explained by the long incubation time which EZH2 inhibitors need before affecting the cell growth. Furthermore, in general the *BAP1*-negative cells demonstrate a slightly more dramatic and faster response to EZH2 inhibition suggesting a potential enhancement of the treatment by *BAP1* loss. This could be, at least partially, explained by the observation that *BAP1*-negative UM tumors tend to have higher *EZH2* expression compared to *BAP1*-positive UM tumors.

Previous studies have already established the potency and interesting therapeutic potential of combined EZH2 and HDAC inhibition in various malignancies [22-24]. Moreover, HDAC inhibition as single treatment or in combination has already been demonstrated to be an interesting strategy for treating UM [27, 28, 33]. Although growth retardation by EZH2-inhibitors takes time, i.e. several replication rounds, at early time points EZH2-inhibition sensitizes the UM cells for HDAC inhibition. We show here for the first time that combined inhibition of EZH2 and HDAC results in a synergic reduction in UM cell growth. Underlying these synergistic effects most likely is the induction of cell death in the combinatory treated cells. In accordance with previous studies, results in this study show increased levels of pro-apoptotic *BIM* and *NOXA* upon dual inhibition of EZH2 and HDACs, correlating with increased *FOXO1* and *FOXO3* mRNA levels, potentially underlying the increase in cell death and the synergism [29]. Although we cannot exclude additional pathways leading to the enhanced apoptosis, previous studies using HDAC inhibitors, including quisinostat, have shown that the increased cell death is at least partly dependent on the enhanced FOXO protein expression [34, 35]. Therefore, we propose that EZH2 inhibition in combination with other compounds, e.g. an HDAC inhibitor like Quisinostat, can provide a useful treatment alternative for metastasized UM.

Acknowledgements

The authors like to thank Dr. Bruce Ksander, Dr. Martine Jager, Dr. Gré Luyten, Dr. Sergio Roman-Roman and Dr. Fariba Nemati for providing the uveal melanoma cell lines. We thank Jinfeng Cao for the gift of GSK503 and UNC1999.

Competing financial interests

The authors declare no competing financial interests.

References

1. Shah SU, Mashayekhi A, Shields CL, Walia HS, Hubbard GB, 3rd, Zhang J, Shields JA. Uveal metastasis from lung cancer: clinical features, treatment, and outcome in 194 patients. Ophthalmology. 2014; 121: 352-7. doi: 10.1016/j.ophtha.2013.07.014.
2. Singh AD, Bergman L, Seregard S. Uveal melanoma: epidemiologic aspects. Ophthalmol Clin North Am. 2005; 18: 75-84, viii. doi: 10.1016/j.ohc.2004.07.002.
3. Augsburger JJ, Correa ZM, Shaikh AH. Effectiveness of treatments for metastatic uveal melanoma. Am J Ophthalmol. 2009; 148: 119-27. doi: 10.1016/j.ajo.2009.01.023.
4. Kivela T, Eskelin S, Kujala E. Metastatic uveal melanoma. Int Ophthalmol Clin. 2006; 46: 133-49. doi:
5. Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. Cancer. 1993; 71: 811-9. doi:
6. Kilic E, van Gils W, Lodder E, Beverloo HB, van Til ME, Mooy CM, Paridaens D, de Klein A, Luyten GP. Clinical and cytogenetic analyses in uveal melanoma. Invest Ophthalmol Vis Sci. 2006; 47: 3703-7. doi: 10.1167/iovs.06-0101.
7. Prescher G, Bornfeld N, Horsthemke B, Becher R. Chromosomal aberrations defining uveal melanoma of poor prognosis. Lancet. 1992; 339: 691-2. doi:
8. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. Lancet. 1996; 347: 1222-5. doi:
9. Harbour JW, Onken MD, Roberson ED, Duan S, Cao L, Worley LA, Council ML, Matatall KA, Helms C, Bowcock AM. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science. 2010; 330: 1410-3. doi: 10.1126/science.1194472.
10. van Essen TH, van Pelt SI, Versluis M, Bronkhorst IH, van Duinen SG, Marinkovic M, Kroes WG, Ruivenkamp CA, Shukla S, de Klein A, Kilic E, Harbour JW, Luyten GP, et al. Prognostic parameters in uveal melanoma and their association with BAP1 expression. Br J Ophthalmol. 2014; 98: 1738-43. doi: 10.1136/bjophthalmol-2014-305047.
11. LaFave LM, Beguelin W, Koche R, Teater M, Spitzer B, Chramiec A, Papalexi E, Keller MD, Hricik T, Konstantinoff K, Micol JB, Durham B, Knutson SK, et al. Loss of BAP1 function leads to EZH2-dependent transformation. Nat Med. 2015; 21: 1344-9. doi: 10.1038/nm.3947.
12. Kim KH, Roberts CW. Targeting EZH2 in cancer. Nat Med. 2016; 22: 128-34. doi: 10.1038/nm.4036.
13. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature. 2011; 469: 343-9. doi: 10.1038/nature09784.
14. Di Croce L, Helin K. Transcriptional regulation by Polycomb group proteins. Nat Struct Mol Biol. 2013; 20: 1147-55. doi: 10.1038/nsmb.2669.
15. Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT, Bender W, Kingston RE. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell. 1999; 98: 37-46. doi: 10.1016/S0092-8674(00)80604-2.
16. Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer. 2006; 6: 846-56. doi: 10.1038/nrc1991.
17. Yoo KH, Hennighausen L. EZH2 methyltransferase and H3K27 methylation in breast cancer. Int J Biol Sci. 2012; 8: 59-65. doi:
18. Xu K, Wu ZJ, Groner AC, He HH, Cai C, Lis RT, Wu X, Stack EC, Loda M, Liu T, Xu H, Cato L, Thornton JE, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science. 2012; 338: 1465-9. doi: 10.1126/science.1227604.
19. Schoumacher M, Le Corre S, Houy A, Mulugeta E, Stern MH, Roman-Roman S, Margueron R. Uveal melanoma cells are resistant to EZH2 inhibition regardless of BAP1 status. Nat Med. 2016; 22: 577-8. doi: 10.1038/nm.4098.

20. LaFave LM, Beguelin W, Koche R, Teater M, Spitzer B, Chramiec A, Papalexi E, Keller MD, Hricik T, Konstantinoff K, Micol JB, Durham B, Knutson SK, et al. Uveal melanoma cells are resistant to EZH2 inhibition regardless of BAP1 status Reply. Nature Medicine. 2016; 22: 578-9. doi: 10.1038/nm.4094.
21. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013; 6: pl1. doi: 10.1126/scisignal.2004088.
22. Grinshtein N, Rioseco CC, Marcellus R, Uehling D, Aman A, Lun X, Muto O, Podmore L, Lever J, Shen Y, Blough MD, Cairncross GJ, Robbins SM, et al. Small molecule epigenetic screen identifies novel EZH2 and HDAC inhibitors that target glioblastoma brain tumor-initiating cells. Oncotarget. 2016; 7: 59360-76. doi: 10.18632/oncotarget.10661.
23. Takashina T, Kinoshita I, Kikuchi J, Shimizu Y, Sakakibara-Konishi J, Oizumi S, Nishimura M, Dosaka-Akita H. Combined inhibition of EZH2 and histone deacetylases as a potential epigenetic therapy for non-small-cell lung cancer cells. Cancer Sci. 2016; 107: 955-62. doi: 10.1111/cas.12957.
24. Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, Ustun C, Rao R, Fernandez P, Chen J, Balusu R, Koul S, Atadja P, et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood. 2009; 114: 2733-43. doi: 10.1182/blood-2009-03-213496.
25. Venugopal B, Baird R, Kristeleit RS, Plummer R, Cowan R, Stewart A, Fourneau N, Hellemans P, Elsayed Y, McClue S, Smit JW, Forslund A, Phelps C, et al. A phase I study of quisinostat (JNJ-26481585), an oral hydroxamate histone deacetylase inhibitor with evidence of target modulation and antitumor activity, in patients with advanced solid tumors. Clin Cancer Res. 2013; 19: 4262-72. doi: 10.1158/1078-0432.CCR-13-0312.
26. van der Ent W, Burrello C, Teunisse AF, Ksander BR, van der Velden PA, Jager MJ, Jochemsen AG, Snaar-Jagalska BE. Modeling of human uveal melanoma in zebrafish xenograft embryos. Invest Ophthalmol Vis Sci. 2014; 55: 6612-22. doi: 10.1167/iovs.14-15202.
27. Heijkants R, Willekens K, Schoonderwoerd M, Teunisse A, Nieveen M, Radaelli E, Hawinkels H, Marine JC, Jochemsen A. Combined inhibition of CDK and HDAC as a promissing therapeutic strategy for both cutaneous and uveal metastatic melanoma. Oncotarget. 2017; 9: 14. doi:
28. Landreville S, Agapova OA, Matatall KA, Kneass ZT, Onken MD, Lee RS, Bowcock AM, Harbour JW. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. Clin Cancer Res. 2012; 18: 408-16. doi: 10.1158/1078-0432.CCR-11-0946.
29. Huang JP, Ling K. EZH2 and histone deacetylase inhibitors induce apoptosis in triple negative breast cancer cells by differentially increasing H3 Lys(27) acetylation in the BIM gene promoter and enhancers. Oncology Letters. 2017; 14: 5735-42. doi: 10.3892/ol.2017.6912.
30. Gallagher SJ, Gunatilake D, Beaumont KA, Sharp DM, Tiffen JC, Heinemann A, Weninger W, Haass NK, Wilmott JS, Madore J, Ferguson PM, Rizos H, Hersey P. HDAC inhibitors restore BRAF-inhibitor sensitivity by altering PI3K and survival signalling in a subset of melanoma. Int J Cancer. 2017. doi: 10.1002/ijc.31199.
31. Feng W, Cai D, Zhang B, Lou G, Zou X. Combination of HDAC inhibitor TSA and silibinin induces cell cycle arrest and apoptosis by targeting survivin and cyclinB1/Cdk1 in pancreatic cancer cells. Biomed Pharmacother. 2015; 74: 257-64. doi: 10.1016/j.biopha.2015.08.017.
32. Robertson AG, Shih J, Yau C, Gibb EA, Oba J, Mungall KL, Hess JM, Uzunangelov V, Walter V, Danilova L, Lichtenberg TM, Kucherlapati M, Kimes PK, et al. Integrative Analysis Identifies Four Molecular and Clinical Subsets in Uveal Melanoma. Cancer Cell. 2017; 32: 204-20 e15. doi: 10.1016/j.ccell.2017.07.003.

33. Carol H, Gorlick R, Kolb EA, Morton CL, Manesh DM, Keir ST, Reynolds CP, Kang MH, Maris JM, Wozniak A, Hickson I, Lyalin D, Kurmasheva RT, et al. Initial testing (stage 1) of the histone deacetylase inhibitor, quisinostat (JNJ-26481585), by the Pediatric Preclinical Testing Program. Pediatr Blood Cancer. 2014; 61: 245-52. doi: 10.1002/pbc.24724.
34. Laporte AN, Poulin NM, Barrott JJ, Wang XQ, Lorzadeh A, Vander Werff R, Jones KB, Underhill TM, Nielsen TO. Death by HDAC Inhibition in Synovial Sarcoma Cells. Mol Cancer Ther. 2017; 16: 2656-67. doi: 10.1158/1535-7163.MCT-17-0397.
35. Pei Y, Liu KW, Wang J, Garancher A, Tao R, Esparza LA, Maier DL, Udaka YT, Murad N, Morrissy S, Seker-Cin H, Brabetz S, Qi L, et al. HDAC and PI3K Antagonists Cooperate to Inhibit Growth of MYC-Driven Medulloblastoma. Cancer Cell. 2016; 29: 311-23. doi: 10.1016/j.ccell.2016.02.011.
36. Chen PW, Murray TG, Uno T, Salgaller ML, Reddy R, Ksander BR. Expression of MAGE genes in ocular melanoma during progression from primary to metastatic disease. Clin Exp Metastasis. 1997; 15: 509-18. doi:
37. Luyten GP, Naus NC, Mooy CM, Hagemeijer A, Kan-Mitchell J, Van Drunen E, Vuzevski V, De Jong PT, Luider TM. Establishment and characterization of primary and metastatic uveal melanoma cell lines. Int J Cancer. 1996; 66: 380-7. doi: 10.1002/(SICI)1097-0215(19960503)66:3<380::AID-IJC19>3.0.CO;2-F.
38. Amirouchene-Angelozzi N, Nemati F, Gentien D, Nicolas A, Dumont A, Carita G, Camonis J, Desjardins L, Cassoux N, Piperno-Neumann S, Mariani P, Sastre X, Decaudin D, et al. Establishment of novel cell lines recapitulating the genetic landscape of uveal melanoma and preclinical validation of mTOR as a therapeutic target. Mol Oncol. 2014; 8: 1508-20. doi: 10.1016/j.molonc.2014.06.004.
39. Amirouchene-Angelozzi N, Frisch-Dit-Leitz E, Carita G, Dahmani A, Raymondie C, Liot G, Gentien D, Nemati F, Decaudin D, Roman-Roman S, Schoumacher M. The mTOR inhibitor Everolimus synergizes with the PI3K inhibitor GDC0941 to enhance anti-tumor efficacy in uveal melanoma. Oncotarget. 2016; 7: 23633-46. doi: 10.18632/oncotarget.8054.

Methods

Cell culture growth and viability assays

The UM cell lines MEL202, OMM2.5 and OMM1 were cultured in a mixture of RPMI and DMEM-F12 (1:1 ratio), supplemented with 10% fetal calf serum (FCS). Cell lines OMM2.5 and MEL202 were kindly provided by B Ksander [36]. OMM1 cells were kindly provided by GPM Luyten [37]. Establishment of the UM cell lines MM28, MM66, MP38, XMP46 and MP65 has been described [38] and these cells were maintained in IMDM containing 20% FCS. All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

For short term growth assay the cells were seeded in triplicate, in 96-well format and incubated for 5 days. Cell survival was determined via the Cell Titre-Blue Cell Viability assay (Promega, Fitchburg, WI, USA); fluorescence was measured in a microplate reader (Victor, Perkin Elmer, San Jose, CA, USA).

Tazemetostat, EPZ011989, GSK503, UNC1999 (all purchased from Selleck Chemicals, Houston, TX USA) and Quisinostat (Johnson and Johnson, New Brunswick, NJ, USA) were used at concentrations indicated in the figure legends.

Six UM cell lines were cultured in the continuous presence of EZH2 inhibitors (2 µM Tzms (Tazemetostat) or 3 µM EPZ (EZP011989)). Cell were seeded sparsely in a 6-well format, ranging from $5x10^4$ to $1.2x10^5$ cells/well, depending on the cell line. When the confluency reached 80-90% in the DMSO treated cells all conditions were counted in duplicate, using a Bűrker chamber, and re-seeded with the same initial density to continue the assay. Cells were provided with fresh medium/drugs every 2-3 days.

Western blot analysis

After incubation with drugs as indicated cells were harvested in Laemmli sample buffer. Bradford Ultra (Expedeon, San Diego, Ca USA) was used according to manufacturer's protocol to determine protein concentrations. Equal protein amounts were separated using SDS-PAGE and blotted onto polyvinylidene fluoride transfer membranes (Millipore, Darmstadt, Germany). After blocking in TBST (10 mM Tris-HCl pH8.0, 150 mM NaCl, 0.2% Tween 20) containing 10% milk, membranes were incubated with the following primary antibodies diluted in TBST/5% BSA (H3K27me3 (39155, Active Motif, Carlsbad, CA, USA), USP7 (A300-033A, Bethyl Laboratories, Montgomery, TX, USA), PARP (9542, Cell Signalling Technology, Beverly, MA, USA), Ac-H3 (06-599, Millipore), BIM (2819, Cell Signalling Technology, Beverly, MA, USA) or Vinculin (hVIN-1/V9131,

Sigma-Aldrich, St Louis, MO, USA)) and appropriate HRP-conjugated secondary antibodies (Jackson Laboratories, Bar Harbor, ME, USA). Bands were visualized using chemoluminescence and visualized by exposure to X-ray film.

Flow cytometry

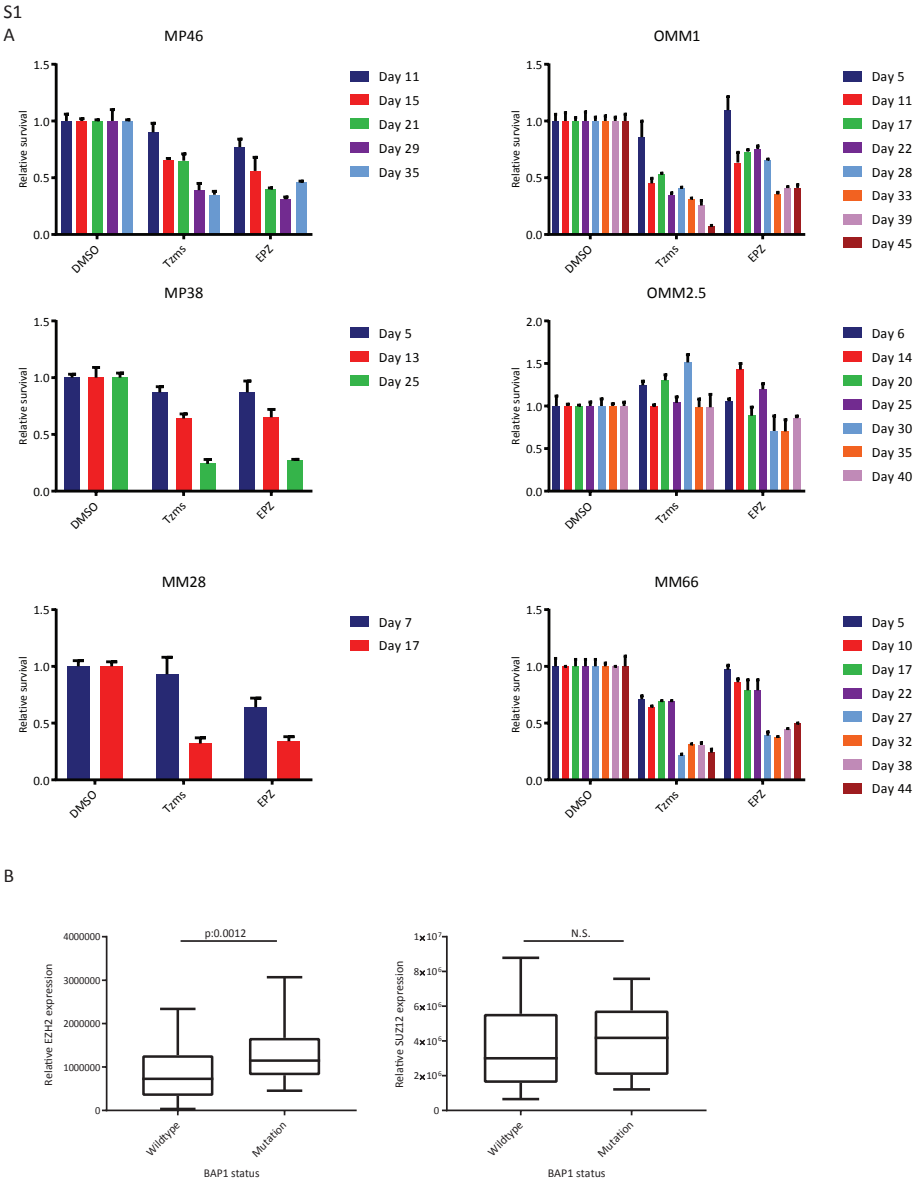
Cells were harvested for cell cycle analysis by trypsinization, washed twice in PBS and fixed in ice cold 70% ethanol. After fixation, cells were washed in PBS containing 2% FCS and resuspended in PBS containing 2% FCS, 50 µg/ml RNAse and 50 µg/ml propidium iodide (PI). Flow cytometry analysis was performed using the BD LSR II system (BD Biosciences, San Diego, CA, USA).

Quantitative PCR

MM28, MP38 and MP46 cell were incubated for 48 hours with 4 µM Tazemetostat, 40 nM Quisinostat or a combination. The SV total RNA isolation kit (Promega, Fitchburg, WI, USA) was used to extract and purify RNA, from which cDNA was synthesized using the reverse transcriptase reaction mixture as indicated by Promega. SYBR green mix (Roche Diagnostics, Indianapolis, IN, USA) was used to perform qPCR in a C1000 touch Thermal Cycler (Bio-Rad laboratories, Hercules, CA, USA). Relative expression of target BIM (Fw: CATCGCGGTATTCGGTTC and Rv: GCTTGCCATTGGTCTTTTT), NOXA (Fw: ACTGTTCGTGTTCAGCTC and Rv: GTAGCACACTCGACTTCC), Survivin (Fw: AGCCCTTTCTCAAGGACCA and Rv: CAGCTCCTTGAAGCAGAAGAA), FOXO1 (Fw: ATGTGTTGCCCAACCAAAGC and Rv: TGCTTCTCTCAGTTCCTGCTG), FOXO3 (Fw: GCGTGCCCTACTTCAAGGAT and Rv: GCTCTTGCCAGTTCCCTCAT) and FOXO4 (Fw: TGCCCAGATCTACGAGTGGA and Rv: GGGTTCAGCATCCACCAAGA) was determined corrected for the housekeeping genes CAPNS1 (Fw: ATGGTTTTGGCATTGACACATG and Rv: GCTTGCCTGTGGTGTCGC), RPS11 (Fw: AAGCAGCCGACCATCTTTCA and Rv: CGGGAGCTTCTCCTTGCC) and SRPR (Fw: CATTGCTTTTGCACGTAACCAA and Rv: ATTGTCTTGCATGCGGCC). Per cell line the average relative expression was determined by setting the untreated at 1.

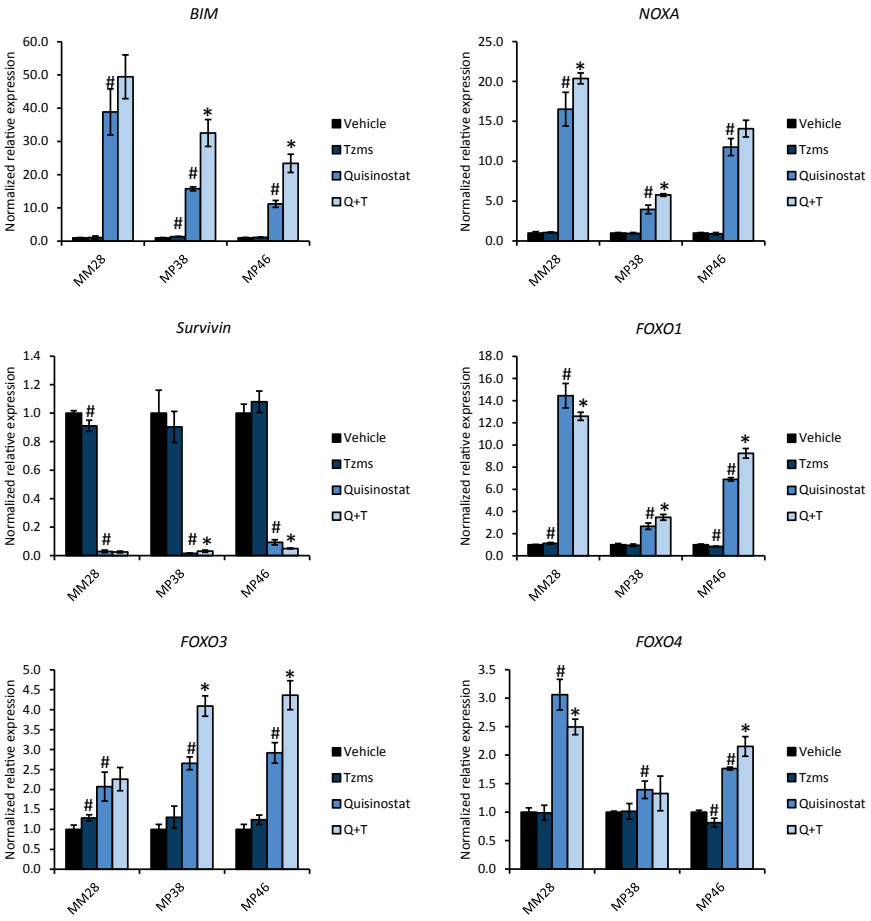
Statistical analysis

To determine significance of changes between two groups a student's t-test was used. P values of 0.05 or lower were considered to be significant.



Supplementary figure 1. Effects of EZH2 inhibition on long-term and in combination with HDAC inhibition on BAP1-positive and -negative uveal melanoma cell lines. (**A**) Extended version of Fig. 1B, showing all time points at which control reached 80-90% confluency and the relative cell survival in the corresponding EZH2 inhibitor treated cells was determined. Long-term growth assays were stopped when cells no longer proliferate or after 40-45 days. (**B**) Comparison of *EZH2* and *SUZ12* expression between UM tumors with diploid or a shallow deleted BAP1.[21]

S2



Supplementary figure 2. Effects of combined EZH2 and HDAC inhibition on *BIM, NOXA, Survivin, FOXO1, FOXO3 and FOXO4* mRNA levels. Normalized relative expression of BIM and NOXA upon 48 hours incubation of 4 μM Tazemetostat (Tzms), 40 nM Quisinostat (Q) or combined. Hashtag (#) indicates significant differences between vehicle and Tazemetostat or Quisinostat treated samples. Asterisk (*) indicates significant differences between the combined treated samples compared to both single treatments.