Overcoming clinical resistance to EZH2 inhibition using rational epigenetic combination therapy

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Abstract

Essential epigenetic dependencies have become evident in many cancers. Based on functional antagonism between BAF/SWI/SNF and PRC2 in *SMARCB1*-deficient sarcomas, we and colleagues recently completed the clinical trial of the EZH2 inhibitor tazemetostat, leading to its FDA approval. However, the principles of tumor response to epigenetic therapy in general, and tazemetostat in particular, remain unknown. Using functional genomics of patient tumors and diverse experimental models, we define molecular mechanisms of tazemetostat resistance in *SMARCB1*-deficient sarcomas and rhabdoid tumors. We found distinct classes of acquired mutations that converge on the RB1/E2F axis and decouple EZH2-dependent differentiation and cell cycle control. This allows tumor cells to escape tazemetostat-induced G1 arrest despite EZH2 inhibition, and suggests a general mechanism for effective EZH2 therapy. Thus, we develop combination strategies to circumvent tazemetostat resistance using cell cycle bypass and synthetic lethal targeting, and provide prospective biomarkers for therapy stratification. This offers a paradigm for rational epigenetic combination therapy suitable for immediate translation to clinical trials for epithelioid sarcomas, rhabdoid tumors, and other epigenetically dysregulated cancers.

Significance: Genomic studies of patient tumors and cell lines identify mutations converging on a common pathway that is essential for response to EZH2 inhibition. Resistance mutations decouple drug-induced differentiation from cell cycle control. We identify complementary epigenetic combination strategies to overcome resistance and improve response, supporting their investigation in clinical trials.

Introduction

Unlike conventional cytotoxic chemotherapy, epigenetic therapy offers the ability to target cancer-specific dependencies with increased specificity and reduced toxicity. This is especially true for cancers caused by genetic mutations of transcriptional regulators, such as the chromatin remodeling BAF/SWI/SNF (Brg/Brahma-associated factors) complex that is mutated in more than 20% of human cancers (1). For example, malignant rhabdoid tumors (MRT) and epithelioid sarcomas (ES) are lethal tumors of children and young adults caused by inactivating mutations of *SMARCB1/SNF5/INI1/BAF47*, one of the core BAF/SWI/SNF complex subunits (2-4). Loss of normal BAF function can confer a dependency on the Polycomb Repressive Complex 2 (PRC2) and its methyltransferase EZH2. This dependency results from epigenetic antagonism between the two complexes, in which normal BAF activity evicts PRC2 from tumor suppressor loci (5, 6). This discovery has led to the development of multiple inhibitors of EZH2 (7-9), several of which have now entered clinical trials (10). However, despite the potential of such targeted epigenetic therapies, the principles governing their effective application remain unknown. Intrinsic and acquired resistance limits their use as monotherapies (11, 12).

A recent clinical trial led to the FDA approval of the EZH2 methyltransferase inhibitor tazemetostat (TAZ) as the first targeted therapy for *SMARCB1*-deficient epithelioid sarcomas (13). However, only 15% of patients showed objective clinical responses, with most epithelioid sarcomas being resistant to TAZ. Ongoing clinical trials in patients with rhabdoid tumors have shown similar results, with only a subset of brain rhabdoid tumor patients exhibiting objective responses to TAZ, while extracranial rhabdoid tumors appear to be uniformly resistant (14). Recent studies have nominated the histone methyltransferase NSD1 as a regulator of TAZ susceptibility in rhabdoid tumor cells, but the clinical relevance of this mechanism is currently not known (15). In all, the outcomes for most patients with MRT and ES remain dismal, and the mechanisms of clinical response and resistance to EZH2 inhibition remain unknown. This hinders our ability to stratify treatment using prospective biomarkers to identify patients who may benefit from TAZ and to develop effective combination therapies with improved and durable benefits for patients.

Here, we define the key requirements for effective epigenetic therapy in diverse *SMARCB1*-deficient epithelioid sarcomas and rhabdoid tumors *in vivo*. Using comparative genomic analyses of clinical trial patients treated with TAZ, we identify multiple acquired mutations that

cause therapy resistance. Using functional genomic approaches, we show that resistance mechanisms converge on a common RB1/E2F axis that integrates control of tumor cell division and differentiation. This organizes patient resistance mutations into a general framework, allowing us to develop rational combination therapies to circumvent TAZ resistance. Using diverse patient-derived ES and MRT cell lines and mouse xenografts, we demonstrate cell cycle bypass and synthetic lethal treatment strategies suitable for immediate translation to combination clinical trials for patients.

Results

Patient tumor sequencing reveals diverse resistance mutations

To identify mutations associated with clinical resistance to TAZ, we performed targeted gene sequencing of patient tumors using MSK-IMPACT, which is based on a panel of over 500 genes recurrently mutated in diverse cancer types (16). We analyzed 33 tumor specimens from 20 patients treated as part of the recent TAZ clinical trial (13), and identified somatic tumor mutations in matched pre- and post-treatment specimens (**Supplementary Tables 1 and 2**). We found distinct sets of somatic mutations in responding and non-responding tumors, with nearly all mutations, apart from *SMARCB1* loss itself, being exclusive to either TAZ-responsive or TAZ-resistant tumors (**Figure 1A, top panel**). Strikingly, we observed two tumors which initially responded to TAZ based on radiographic imaging but later developed clinical resistance (**Figure 1A, bottom panel, 1B**). Targeted sequencing of the resistant tumors revealed two newly acquired somatic mutations: homozygous missense mutation of *EZH2* ($EZH2^{Y666N}$) in the Patient 3 tumor specimen, and biallelic loss of function mutation of *RB1*, including a hemizygous deletion and a frame shift mutation ($RB1^{del}$) in the remaining allele, in the Patient 15 tumor specimen. We confirmed both mutations using RNA-seq of the respective tumor specimens (**Figure S1A-B**). Since one mutation affected *EZH2* directly, and the other involved the known EZH2 target *RB1* (17), we hypothesized that both mutations were responsible for TAZ resistance in their respective patients.

First, we investigated the $EZH2^{Y666N}$ mutation. Past studies using forward genetic screens in lymphoma cell lines have identified putative resistance mutations within both the N-terminal D1 domain and the catalytic SET domain of EZH2, both of which are predicted to interact with S-adenosyl methionine (SAM)-competitive EZH2 inhibitors such as TAZ (18, 19). One such SET

domain mutation previously identified in cell lines is *EZH2*^{Y661D}, with Y661 corresponding to Y666 in isoform 2 of *EZH2*, the isoform referred to in this study, and thus concordant with the mutation we observed in the Patient 3 tumor (18).

Based on the atomic resolution structure of a pyridone-based EZH2 inhibitor bound to the *Anolis carolinensis* EZH2 (PDB 5IJ7) (20), we reasoned that residue Y666 in human EZH2 may form a critical part of the TAZ binding site and that its mutation can prevent TAZ from binding to the SET domain (**Figure 1C**). To test *EZH2*^{Y666N} as a resistance allele, we expressed doxycycline-inducible *EZH2*^{Y666N} in *SMARCB1*-deficient G401 rhabdoid tumor cells. We observed that *EZH2*^{Y666N} expressing clones are resistant to TAZ as compared to cells expressing equal levels of wild-type *EZH2* by assessing both cell viability (**Figure 1D**) and cell morphology (**Figure S1C**). The resistance phenotype of *EZH2*^{Y666N}-expressing cells depends on the intact catalytic activity of the SET domain, as a compound mutant combining the catalytically inactive triple mutant *EZH2*^{F672I,H694A,R732K} (*EZH2*^{CatMut}) with the Y666N mutation, termed *EZH2*^{QuadMut}, did not confer resistance to TAZ (**Figure S1D**). We also observed that *EZH2*^{Y666N} confers resistance to the dual EZH1/2 inhibitor valemetostat (21), consistent with putative resistance to SAM-competitive, pyridone-based EZH2 inhibitors (**Figure S1E**). This also suggests that combined inhibition of EZH2 and EZH1 may not overcome this type of acquired EZH2 inhibitor resistance.

Previous studies found that lymphoma cells resistant to EZH2 inhibitors remained susceptible to the inhibition of the non-enzymatic PRC2 subunit EED (22), including those with mutations in the *EZH2* SET domain (*EZH2*^{C663Y} and *EZH2*^{Y726F}) and the D1 domain (19, 23). We therefore hypothesized that TAZ resistance conferred by *EZH2*^{Y666N} could be overcome by PRC2 inhibitors that do not bind to EZH2. Indeed, we found that the allosteric EED inhibitor MAK683 overcomes *EZH2*^{Y666N}-mediated resistance (24), demonstrating that these cells remain generally susceptible to PRC2 inhibition (**Figure 1D**). EED inhibition may thus be an effective strategy to overcome acquired TAZ resistance mutations in *EZH2*.

RB1 **loss allows escape from cell cycle arrest despite effective EZH2 inhibition**

Past work has shown that *EZH2* is a direct target of repression by RB1/E2F (17, 25). This suggests that acquired *RB1* loss observed upon TAZ treatment may confer resistance to EZH2 inhibition by increasing EZH2 expression. To test *RB1*^{del} as a TAZ resistance allele, we used CRISPR/Cas9 genome editing to generate biallelic *RB1*^{del} mutations in G401 cells, as compared

to isogenic *RB1*-wild type control cells produced by targeting the safe harbor locus *AAVS1*. We confirmed absence of RB1 protein expression in two independent clones using Western blotting, and found that *RB1*^{del} cells were indeed resistant to TAZ (**Figure 1E**).

Despite *EZH2* being a known target gene of RB1/E2F, we were surprised to observe that *RB1*^{del} G401 cells showed similar morphological changes upon TAZ treatment (**Figure S2A**) to those previously reported for TAZ-treated *RB1*^{WT} G401 cells (7). To define the effects of TAZ on *RB1*^{del} cells more precisely, we performed RNA-seq of isogenic G401 *RB1*^{del} and wildtype *AAVS1*-control cells, treated with either 10 µM TAZ or DMSO control for 11 days, based on an established treatment regimen to model EZH2 inhibition *in vitro* (7). As predicted, we observed that *EZH2* mRNA and protein levels remained high in TAZ-treated *RB1*^{del} cells, unlike in *RB1*^{WT} cells (**Figure 2A-C, S2B**). However, EZH2 inhibition induced significant upregulation of hundreds of genes in both *RB1*^{WT} and *RB1*^{del} cells, including upregulation of known PRC2 target genes (**Figure 2A-F**).

Importantly, trimethylation of the EZH2 substrate H3K27 was substantially reduced by TAZ regardless of *RB1* status (**Figure S2B**). This indicates that despite persistent EZH2 expression, EZH2 methyltransferase activity is effectively inhibited by TAZ despite *RB1* loss. Gene set enrichment analysis (GSEA) showed that all three clones significantly upregulated multiple gene sets upon TAZ treatment (**Supplementary Table S3**, **Figure S2C**). This included gene sets associated with cell differentiation such as epithelial-to-mesenchymal transition (EMT; **Figure S2F**). This is reminiscent of recent observations that re-expression of *SMARCB1* in G401 cells can lead to a mesenchymal chromatin state (26), and is consistent with the idea that PRC2 inhibition may allow BAF to re-activate a more developmentally normal gene expression state. Taken together, these findings indicate that *RB1* loss-induced TAZ resistance is independent of EZH2.

In addition to *EZH2*, additional RB1/E2F target genes were also upregulated in TAZ-treated *RB1*^{del} cells compared to TAZ-treated *RB1*^{WT} control cells (**Figure S2D-E**). Given the function of RB1 in the regulation of the G1/S cell cycle checkpoint, we hypothesized that *RB1* loss could allow cells to escape TAZ-induced cell cycle arrest. Flow cytometry cell cycle analysis showed that G401 cells treated with 1 µM TAZ arrest at the G1/S checkpoint, as reported previously (7). However, *RB1*^{del} cells exhibited a significant reduction in the proportion of cells in G1 phase upon TAZ treatment (50% of TAZ-treated *RB1*^{WT} cells versus 31% and 28% for *RB1*^{del} E1 and

F2 clones, respectively), with a corresponding increase of the proportion of cells remaining in S and G2/M phases (**Figure 2G**). In agreement with this, we observed persistent mRNA expression of S/G2/M-phase-associated *CCNA2*, *CDK2*, and *AURKB* genes in *RB1*^{del} cells upon TAZ treatment (**Figure 2A-C**). This is despite upregulation of the CDK4/6 inhibitor *CDKN2A* (p16), a known PRC2 target in MRT (5, 27, 28), upon TAZ treatment in both *RB1*^{WT} and *RB1*^{del} cells (**Figure 2A-C**). We confirmed persistent maintenance of S-phase cyclin A2 (CCNA2) protein levels despite p16 upregulation using Western blotting (**Figure 2H**). Together, these results show that *RB1* loss is sufficient to evade TAZ-induced cell cycle arrest at the G1/S restriction point despite maintaining the expected global transcriptional response to EZH2 inhibition, including upregulation of cell cycle inhibitor genes.

Intact RB1/E2F axis is required for TAZ susceptibility

The requirement for *RB1* expression in the therapeutic response to TAZ suggests that an intact RB1/E2F axis may be a general requirement for effective EZH2 inhibitor therapy. This would predict that other genetic and epigenetic perturbations to the RB1/E2F axis, beyond *RB1* loss itself, would similarly confer escape from TAZ-induced cell cycle arrest. Analysis of our TAZ clinical trial treatment cohort revealed one patient tumor specimen with primary resistance to TAZ with intact *RB1* but inactivating mutations of both *CDKN2A* and *CDKN2B* (**Figure 1A**, **Supplementary Tables 1-2**), both of which are known to inhibit CDK4/6-mediated phosphorylation of RB1. Two additional specimens had missense mutations in *ANKRD11*: One tumor with primary resistance to TAZ and another which initially responded but later progressed on treatment, at which point a newly acquired *ANKRD11* mutation was detected (**Figure 1A**-Patients 9 and 16, respectively). *ANKRD11* is a known TP53 cofactor and putative tumor suppressor that contributes to TP53-mediated expression of pan-CDK inhibitor *CDKN1A* (29-31). *CDKN1A* itself is also known to be a PRC2 target in tumors (32-34), although its role in the response to EZH2 inhibition in *SMARCB1*-deficient sarcomas is currently unknown. These results converge on the dysregulation of the RB1/E2F axis as a mechanism of evasion of TAZ-induced cell cycle arrest.

To investigate the functional determinants of tumor cell response to TAZ, we first analyzed the response to TAZ of seven MRT and four ES cell lines in which we confirmed loss of SMARCB1 protein expression in all ES and MRT cell lines using Western blotting, as compared to SMARCB1-expressing HEK293T cells (**Figure S3A**). We classified each line as sensitive or

resistant based on the area under the curve (AUC) of their TAZ dose responses (AUC > 0.3 for sensitive G401, KP-MRT-NS, TTC642, A204, TM8716, KP-MRT-RY cell lines and AUC ≤ 0.3 for resistant ES1, VAESBJ, ES2, EPI544, MP-MRT-AN cell lines; **Figure 3A**). Given that TAZ treatment requires at least 4 days for the cellular reduction of methylated EZH2 substrates and at least 7 days for apparent antiproliferative effects in the rapidly-dividing G401 cell line (7), we confirmed that the apparent TAZ susceptibilities of these MRT and ES cell lines are not correlated with their proliferation rates (Pearson's r = -0.073 and p = 0.81; **Figure S3B**). In agreement with somatic mutations affecting the RB1/E2F axis associated with TAZ resistance in clinical tumor specimens (**Figure 1A**), we found mutations of *CDKN2A* in 4 out of 5 TAZ-resistant cell lines, *CDKN2B* in 2 out of 5 resistant cell lines, *CDKN1A* in 1 out of 5, and *ANKRD11* in 3 out of 5, as compared to no such mutations in any TAZ-sensitive MRT and ES cell lines (**Figure 3B**, **Supplementary Table 4**). While our analysis detected reduction in copy number of *RB1* in TAZ-sensitive KP-MRT-RY cells, manual inspection of sequencing reads within the *RB1* gene revealed lack of homozygous deletion, with presumed retention of *RB1* expression (**Figure S3C**). Thus, mutations associated with the RB1/E2F axis are associated with resistance to TAZ in *SMARCB1*-deficient cell lines and patient tumors.

We next assessed the apparent transcriptional activity of the RB1/E2F axis in patient tumors using quantitative gene expression analysis of TAZ responding and non-responding tumors biopsied before and after TAZ treatment using RNA-seq (**Supplementary Table S1**). Pre-treatment TAZ-resistant tumors exhibited significant enrichment of multiple Gene Ontology (GO) terms associated with the cell cycle and in particular with the S and G2/M phases (**Figure S4A**). Similarly, post-treatment tumors that progressed on TAZ showed increased gene expression of GO terms associated with mitosis, as compared to TAZ-responsive tumors (**Figure S4B**). Indeed, TAZ-resistant tumors exhibited consistently higher expression of S/G2/M-phase-associated genes prior to treatment (**Figure S4C-D**). These findings suggest that in addition to the mutations affecting the RB1/E2F axis associated with TAZ resistance, additional mutations not captured by MSK-IMPACT targeted gene sequencing and/or epigenetic dysregulation, such as putative silencing of tumor suppressor genes like *CDKN1A* or *CDKN2A*, likely contribute to TAZ resistance and the decoupling of RB1/E2F-mediated proliferation and PRC2-regulated differentiation.

Since TAZ-resistant MRT and ES cell lines and patient tumors show distinct mutations and gene expression changes that converge on the RB1/E2F axis, we inquired whether these

perturbations would similarly converge on common prognostic biomarkers of TAZ resistance. Comparative gene expression analysis of untreated *RB1*^{del} cells versus *RB1*^{WT} G401 cells showed a small set of consistently and significantly up- and down-regulated genes in two independent clones (**Figure S5A**). The most substantially and significantly upregulated gene associated with *RB1* loss was *PRICKLE1* (**Figure 3C-D**), which we confirmed to be overexpressed at the protein level in both *RB1*^{del} clones using Western blotting (**Figure 3E**).

In agreement with this, we also found that *PRICKLE1* was among the most differentially expressed genes between 10 pre-treatment patient tumors with response and resistance to TAZ, with *PRICKLE1* expression being higher in TAZ-resistant tumors (mean normalized reads = 10,237 and 300 for resistant and responsive tumors, respectively; Student's t-test p = 0.013; **Figure 3F, S5A**). *PRICKLE1* can control planar cell polarity (PCP), a key cell differentiation pathway, and has previously been implicated as a prognostic biomarker of poor prognosis in breast cancer (35, 36), acute myeloid leukemia (37), and gastric cancer (38, 39). Several other genes encoding PCP pathway factors were also upregulated in TAZ-resistant tumors compared to TAZ-sensitive tumors (**Figure S5A**). We next looked for differentially expressed genes in TAZ-sensitive tumors as potential markers of sensitivity. These included the transcription factor *KLF4* which can control the G1/S transition by regulating *CDKN1A* expression (40, 41) (mean normalized reads = 200 and 2,489 for resistant and responsive tumors, respectively; Student's-test p = 0.03; **Figure S5B, 3G**). Thus, PRICKLE1 and additional factors controlling PCP and integration of RB1/E2F cell cycle and differentiation are potential prognostic pre-treatment biomarkers to identify clinical TAZ resistance and susceptibility of *SMARCB1*-deficient tumors.

Synthetic lethal and cell cycle bypass epigenetic combination strategies overcome tazemetostat resistance

Given that *RB1*^{del} cells are able to bypass cell cycle arrest at the G1/S checkpoint, we reasoned that inhibiting cell cycle kinases downstream of this checkpoint could overcome TAZ resistance. In particular, cell cycle kinases *CDK2* and *AURKB*, which are downregulated by EZH2 inhibition in TAZ-sensitive cells but persistently expressed in TAZ-resistant cells, may offer especially compelling therapeutic targets to overcome TAZ resistance (**Figure 2A-C**). Indeed, we found that the CDK2 inhibitor seleciclib (42), as well as the mitotic kinase Aurora A inhibitor alisertib (43), and Aurora B inhibitor barasertib (44), were able to overcome TAZ resistance in *RB1*^{del} G401 cells (**Figure 4A, Figure S6A-B**). We term this combination strategy cell cycle bypass.

Consistent with the function of CDK4/6 kinases upstream of RB1/E2F, sensitivity to CDK4/6 inhibitors palbociclib and abemaciclib was reduced by *RB1*^{del} mutation (**Figure S6C-D**). In support of the cell cycle bypass strategy for TAZ combination therapy, we observed that patient tumors which progressed on TAZ showed higher expression of *AURKB* mRNA as compared to those that responded (**Figure 3H**). Combined with the high sensitivity of G401 cells to barasertib (**Figure 4A**; half-maximal effective concentration of 6.5 ± 0.5 nM, 5.5 ± 0.6 nM, and 5.9 ± 0.9 nM for *RB1*^{WT}, *RB1*^{del} E1, and F2 clones, respectively), these findings suggest that the cell cycle bypass strategy may effectively overcome TAZ resistance.

We therefore asked whether the combination of TAZ and barasertib would have activity against both TAZ-responsive and TAZ-resistant *SMARCB1*-deficient MRT and ES cell lines, using RPE cells as a *SMARCB1*-proficient control. The effects of the combination of TAZ and barasertib on cell viability did not substantially exceed the effect of barasertib alone at the doses tested (**Figure 4B**; 200 nM TAZ, 8 nM barasertib). However, nearly all cell lines tested, including those resistant to TAZ monotherapy, showed substantial susceptibility to barasertib, with RPE cells displaying the lowest sensitivity (**Figure 4B**).

To test the effects of this combination *in vivo*, we treated a panel of five patient-derived rhabdoid tumor and epithelioid sarcoma xenografts (PDX) in immunodeficient mice (**Supplementary Table S5**), comparing TAZ and barasertib monotherapies with their combination. Importantly, in contrast to the modest reduction of tumor growth and extension of survival of mice with tumors <1,000 mm^3 with TAZ or barasertib alone, PDX mice treated with the combination of TAZ and barasertib showed significant reductions in tumor growth (Vardi *U*-test p = 4.0E-4 and 2.0E-4 for combination versus barasertib or TAZ, respectively; **Figure 4C**) (45). In two of the PDX models, this combination led to tumor regressions (**Figures 4E-F & S8A**). Consistent with this benefit, the combination was also found to significantly increase mean tumor-free animal survival from 65 days (95% confidence interval (CI) = 51-78 days) for barasertib and 67 days (95% CI = 53-81 days) for TAZ to 98 days (95% CI = 84-112 days) for the combination (log-rank test p = 3.3E-3 and 5.8E-3 for combination versus barasertib or TAZ, respectively; **Figure 4D**). These results indicate that the combination of TAZ with a downstream cell cycle inhibitor such as barasertib can improve response and overcome resistance to TAZ in diverse rhabdoid tumors and epithelioid sarcomas *in vivo*.

In addition to distinct cell cycle dynamics of TAZ resistance, we observed that regardless of *RB1* status, TAZ treatment also caused significant increase in expression of *PiggyBac transposable element derived 5* (*PGBD5)* (**Figure 2A-C**). *PGBD5* is a transposase-derived gene with retained nuclease activity in human cells, which has been implicated as a somatic mutator and inducer of double-strand DNA (dsDNA) breaks in childhood solid tumors (46-48). In rhabdoid tumors in particular, PGBD5 was observed to induce sequence-specific mutations and DNA rearrangements, including somatic deletions of *SMARCB1* itself (46). In turn, *PGBD5* expression was both necessary and sufficient to confer a cellular dependency on end-joining DNA repair and ATR kinase signaling (47).

TAZ-induced upregulation of *PGBD5* expression suggests that TAZ treatment may potentiate this synthetic lethal dependency. To test this idea, we used the ATR-selective kinase inhibitor elimusertib, which is currently undergoing clinical trials in patients with solid tumors, including patients with PGBD5-expressing tumors such as MRT and ES (Clinical Trials Identifier NCT05071209). We found that elimusertib exhibited low-nanomolar potency against $RB1^{WT}$ and $RB1^{del}$ G401 cells *in vitro* (half-maximal effective concentration of 17.6 ± 1.6 nM, 19.2 ± 3.8 nM, and 26.7 ± 3.2 nM for $RB1^{WT}$, $RB1^{del}$ E1 and F2 clones, respectively; **Figure 5A**). We also found that the combination of TAZ and elimusertib exerted greater antitumor effects than either drug alone against diverse MRT and ES cell lines (**Figure 5B**), exhibiting synergy in a subset of the cell lines (**Figure S6E-F**). To determine whether the synergistic elimusertib and TAZ combination antitumor effects were due to increased DNA damage, we used confocal immunofluorescence microscopy to quantify γH2AX phosphorylation, a specific marker of dsDNA breaks (49). In agreement with prior studies (47), untreated G401 cells showed dsDNA breaks associated with baseline *PGBD5* expression (**Figure S10A**). Consistent with TAZ-mediated induction of *PGBD5* expression (**Figure 2A-C**), we found that TAZ treatment alone significantly increased nuclear γH2AX fluorescence (median normalized level = 0.061 versus 0.12, respectively; *t*-test p = 1.7E-8; **Figures 5C & S10B**), and the combination of TAZ and elimusertib induced additional dose-dependent increases in dsDNA break levels than either drug alone (median normalized level = 0.079 versus 0.12 and 0.20, respectively; *t*-test p = 5.1E-3 and 6.5E-4 for 50 and 100 nM TAZ, respectively; **Figure 5D**). Targeting TAZ-potentiated and PGBD5-induced DNA damage using the ATR kinase-selective inhibitor elimusertib was specific, because combination of TAZ with the DNA replication repair CHK1 kinase-selective inhibitor SRA737 showed no increased activity as compared to either drug alone (**Figure S9A-B**). Indeed, TAZ treatment did not induce apparent replication stress, as measured by RPA

phosphorylation (**Figure S9B**), which was also not potentiated by combined CHK1 inhibition with SRA737, in spite of effective suppression of CHK1 auto-phosphorylation (**Figure S9B**). Unlike T-ALL, where EZH2 suppression induces MYCN protein expression and replication stress (50), TAZ treatment of G401 rhabdoid tumor cells failed to increase MYCN protein abundance (**Figure S9C**), in spite of significant upregulation of *MYCN* mRNA (**Figure 2A-C**).

Encouraged by the potent and specific antitumor activity of synthetic lethal combination TAZ therapy *in vitro*, we tested the antitumor activity of elimusertib and TAZ combination using a diverse cohort of MRT and ES PDX mice *in vivo* (**Supplementary Table S5**). The combination of TAZ and elimusertib exceeded the effect of treatment with either drug alone when assessed by tumor measurements (Vardi *U*-test p = 2.0E-2 and 0.19 for combination versus elimusertib or TAZ, respectively; **Figure 5E**) and significantly extended tumor-free survival from 51 days (95% CI = 42-60 days) for elimusertib and 67 days (95% CI = 53-81 days) for TAZ to 99 days (95% CI = 74-123 days) for the combination (log-rank test p = 5.8E-4 and 3.9E-2 for combination versus elimusertib or TAZ, respectively; **Figure 5F**). This was most pronounced for the HYMAD_EPIS_X0004aS1 tumor (**Figures 5G-H & S8B-C**), which exhibited a relatively poor response to TAZ monotherapy, when assessed by tumor growth measurements (Vardi *U*-test p = 2.0E-4 for combination versus elimusertib or TAZ; **Figure 5G**) and tumor-free survival (log-rank test p = 6.2E-3 and 6.3E-5 for combination versus elimusertib or TAZ, respectively; **Figure 5H**). Thus, the combination of EZH2 and ATR inhibition constitutes a synthetic lethal rational combination strategy to improve TAZ clinical response and overcome resistance.

Discussion

What defines effective epigenetic EZH2 inhibition therapy for *SMARCB1*-deficient epithelioid sarcomas and rhabdoid tumors? Our studies of ES patients treated with TAZ demonstrate that effective inhibition of PRC2 enzymatic activity is necessary but not sufficient for durable antitumor effects. Using clinical genomics and transcriptomics, combined with functional genetic studies of more than 15 diverse MRT and ES cell lines and patient-derived tumors *in vitro* and *in vivo*, we propose a general molecular model for effective epigenetic TAZ therapy (**Figure 6**). This model places validated *RB1* and *EZH2* TAZ resistance alleles within the context of a molecular sequence of events required for clinical TAZ response. This model also explains additional mutations associated with TAZ resistance based on the perturbation of each stage of this sequence and provides candidate prognostic biomarkers and therapeutic combination

strategies. We discuss the evidence for this model below, and summarize its novel predictions and implications.

First, TAZ must be able to bind and enzymatically inhibit the EZH2 SET domain (**Figure 6; Step 1**). This inhibition can be blocked by gatekeeper mutations of the EZH2 drug binding site, as observed in lymphoma cell lines (18), and demonstrated for the first time here in an epithelioid sarcoma with clinically acquired *EZH2*^{Y666N} mutation. Such resistance mutations can be overcome by targeting EED, a non-enzymatic PRC2 subunit.

For effective epigenetic TAZ therapy, chromatin remodeling complexes must act on tumor suppressor loci that were aberrantly repressed by PRC2 (**Figure 6; Step 2**). The canonical BAF complex is thought to oppose the activity of the Polycomb Repressive Complex (PRC), associated with its chromatin eviction (5, 6). However, the precise mechanism of eviction of TAZ-inhibited PRC2 in *SMARCB1*-deleted tumors is not fully defined. This may involve TAZ-induced remodeling of BAF and/or PRC2. For example, a recent study of *SMARCA4*-deficient cell lines found that upregulation of the expression of BAF helicase *SMARCA2*, which is under PRC2 control in these cells, is necessary for response to EZH2 inhibition (51). In our study, we observed *RB1*-independent upregulation of the expression of BAF subunits *SMARCA2* and *DPF3* upon TAZ treatment in G401 cells (**Figure S10A**). This suggests that TAZ may impact BAF complex assembly as part of its therapeutic mechanism in *SMARCB1*-deficient tumor cells. However, this also indicates that *SMARCA2* re-expression upon EZH2 inhibition is not sufficient to induce cell cycle arrest in the absence of *RB1* expression. After assessing TAZ-induced gene expression changes in PRC2 subunits, we also observed *RB1*-independent TAZ-induced upregulation of PRC2 subunit *JARID2*, and *RB1*-dependent downregulation of *PHF19*, suggesting that PRC2 composition itself may be affected by TAZ treatment (**Figure S10A**). Additionally, it is unknown whether PRC2 eviction in TAZ-treated cells requires a specific form of the BAF complex, such as the non-canonical *SMARCB1*-deficient ncBAF or GBAF complex described previously (52, 53).

If the activity of specific BAF subtypes is indeed needed to evict PRC2 from chromatin upon EZH2 inhibition, then genetic perturbation of specific BAF subunits may impact tumor response to TAZ. For example, in our patient cohort, we observed one TAZ-sensitive tumor with a truncation in the canonical BAF-specific subunit *ARID1B*, while a TAZ-resistant tumor had a missense mutation in PBAF-specific subunit *ARID2* (**Figure 1A**). We also found *ARID1B* to be

mutated in 2 out of 5 TAZ-responsive cell lines (0 out of 5 TAZ-sensitive cell lines) and *ARID2* to be mutated in 2 out of 5 TAZ-resistant cell lines, though also in 1 out of 5 TAZ-sensitive lines (**Figure 3B**). It is not known whether these mutations affect tumor response to TAZ, and further work will be needed to elucidate the specific mechanism of chromatin de-repression in TAZ-treated tumor cells, and its requirement of specific BAF subunits and complexes.

Effective epigenetic TAZ therapy must also upregulate PRC2-repressed tumor suppressor loci (**Figure 6; Step 3**). In our patient cohort, one TAZ-resistant tumor had deletions of both *CDKN2A* and *CDKN2B* (**Figure 1A**), which can inhibit CDK4/6 from phosphorylating RB1 and are known to be de-repressed by TAZ treatment (7). Our genomic analysis of MRT and ES cell lines also showed that 4 out of 5 TAZ-resistant cell lines tested had apparent loss of *CDKN2A*, with two also having loss of *CDKN2B*, and one having *CDKN1A* loss as well (**Figure 3B**). These mutations may phenocopy *RB1* loss, suggesting that upregulation of these cell cycle inhibitors may be necessary for effective TAZ therapy.

In addition to the preservation of these tumor suppressor loci, transcription factors and coactivators that upregulate their expression must also be intact and expressed for tumor cells to effectively respond to TAZ (**Figure 6; Step 3**). In our patient cohort, 2 TAZ-resistant patient tumors had missense mutations in *ANKRD11* (**Figure 1A**), as did 3 out of 5 TAZ-resistant cell lines. *ANKRD11* is a putative tumor suppressor that exhibits loss of heterozygosity in breast cancer (54), and is recurrently mutated in other cancers (55, 56). *ANRKD11* can cooperate with p53 to upregulate *CDKN1A*, and previous reports have suggested possible risk of cancer development in patients with a constitutional loss of *ANKRD11* (57, 58). While its importance in *SMARCB1*-deficient sarcomas is unknown, its association with TAZ resistance suggests potential causality. We note that its proposed tumor suppressor function would require all alleles to be lost or mutated. Consistent with potential functional significance, EPI544 cells harbor multiple mutations in *ANKRD11*, two with an apparent allele frequency of 1.0 and MP-MRT-AN cells harbor two mutations, both with 0.5 allele frequencies (**Supplementary Table S4**). Interestingly, a recent report described a patient with KBG syndrome, a developmental condition caused by mutation of *ANKRD11*, who also developed a rhabdoid tumor (58). The co-occurrence of these two exceedingly rare conditions in the same patient is consistent with potential functional involvement of *ANKRD11* in rhabdoid tumor development and susceptibility to EZH2 inhibition.

We also identified *KLF4* as a putative marker of susceptibility to TAZ in patient tumors (**Figure 3G**), and found *KLF4* to be mutated in 1 out of 5 TAZ-resistant cell lines (**Figure 3B**). Although KLF4 is a transcription factor with both an activating and repressing functions, its role in *SMARCB1*-deleted tumors is not known. Its expression has long been known to upregulate *CDKN1A*, causing cell cycle arrest at the G1/S checkpoint (40, 59). This suggests that KLF4 may regulate the induction of tumor suppressor genes in response to EZH2 inhibition, such as its bona fide target *CDKN1A*. This may occur through recruitment of BAF to tumor suppressor loci; KLF4 can recruit BAF to target genes to upregulate them (60). Like *ANKRD11*, *KLF4* may thus be a key activator of PRC2-repressed genes (**Figure 5**).

Finally, effective TAZ therapy also requires the function of downstream cell cycle effectors of the relevant tumor suppressor loci (**Figure 6; Step 4**). As we have demonstrated in this study, loss of *RB1* leads to the evasion of TAZ-induced cell cycle arrest, despite effective inhibition of EZH2 activity and sustained transcriptional response to TAZ. A recent genome-wide CRISPR screen also found *RB1* as a top mediator of TAZ resistance (15). This is reminiscent of the necessity of intact *RB1* for therapeutic response to clinical inhibitors of CDK4/6 (61-63). The transcriptional upregulation of hundreds of genes by TAZ in $RB1^{del}$ tumors, including EMT gene sets (**Figure S2C&F**), suggests that these cells are undergoing forced differentiation, even while maintaining proliferation (**Figure 2G-H**). This upregulation of mesenchymal gene sets is consistent with previous work that has shown that PRC2 inhibition, similar to *SMARCB1* re-expression, can drive *SMARCB1*-deficient tumors into a terminally differentiated, mesenchymal-like state (7, 64, 65), possibly recapitulating the normal developmental trajectory of their cells of origin (64). In this way, *RB1* loss and dysregulation of the RB1/E2F axis appear to decouple the regulation of cell fate and identity from its control of cell cycle progression.

In our search for predictive biomarkers of TAZ response, we identified increased expression of the PCP gene *PRICKLE1* to be associated with a deficient RB1/E2F axis and TAZ resistance (**Figure 3C-F**). The molecular mechanism connecting G1/S dysregulation and *PRICKLE1* expression is currently unknown, but the PCP pathway is known to be under cell cycle control (66, 67). This is mediated at least in part by PLK1, which we found to be one of the top upregulated genes in TAZ-resistant tumors (**Figure S5B**). Likewise, deletion of the *Drosophila RB1* homologue *Rbf1* results in the upregulation of several PCP genes, including the *PRICKLE1* homologue *pk* (68). This suggests that dysregulation of the RB1/E2F axis may lead to upregulation of *PRICKLE1* through dysregulation of normal cell cycle control of PCP. Further

work will be needed to define this mechanism, as well as to investigate PRICKLE1 as a clinical biomarker for TAZ resistance.

Finally, our study developed two strategies to circumvent clinical TAZ resistance. First, since dysregulation of the RB1/E2F axis mediates escape from cell cycle arrest at the G1/S checkpoint, we reasoned that cell cycle kinases that function downstream of this checkpoint would remain viable therapeutic targets. This cell cycle bypass strategy is supported by previous work showing that loss of *RB1* can sensitize cancer cells to Aurora kinase inhibition through a primed spindle assembly checkpoint (69). As predicted, $RB1^{del}$ cells remain sensitive to CDK2, AURKA, and AURKB inhibition (**Figures 4A, S6A-B**). We found that MRT and ES cell lines resistant to TAZ, including those with *ANKRD11* and *CDKN1A/2A/2B* mutations were sensitive to barasertib. Most compellingly, we found that the combination of TAZ and barasertib exhibits improved antitumor activity *in vivo* compared to either drug alone.

This is reminiscent of the therapeutic combination mechanism proposed by Sorger and Palmer (70), in which inter-patient and inter-tumor variability in response to individual drugs rather than their pharmacological interactions lead to apparent combined effects. We observed substantial benefit of TAZ and barasertib combination within individual PDX models. This suggests that the improved efficacy of this combination may also result from intra-tumor heterogeneity and tumor evolution *in vivo*, as proposed for combination therapy more than fifty years ago (71, 72). Dual targeting of two different parts of the cell cycle can prevent tumors from evading cell cycle arrest through the presence or acquisition of mutations in cell cycle control genes. For example, cells with a defective G1/S checkpoint that continue to transit through the cell cycle remain sensitive to AURKB inhibition. Further work will be needed to elucidate whether barasertib causes postmitotic endoreduplication due to its disruption of the spindle assembly and/or direct inhibition of the mitotic checkpoint (73, 74).

Our findings also advance synthetic lethal strategy for rational epigenetic TAZ combination therapy due to TAZ-induced expression of *PGBD5*, the putative developmental mutator in rhabdoid and other young-onset solid tumors, due to its induction of dsDNA breaks (46). We found that the DNA damage repair ATR kinase inhibitor elimusertib not only overcomes RB1/E2F axis-mediated resistance, but in combination with TAZ, also exerts synergistic anti-tumor effects *in vitro* and *in vivo*. TAZ-induced upregulation of PGBD5 is associated with the induction of DNA damage. It is possible that the enhanced sensitivity to ATR inhibition due to

the increased requirements for DNA repair also depends on the intrinsic variation in DNA damage repair signaling among different tumor subtypes. This may also be due to the variation in the expression and activity of PGBD5 nuclease activity among tumors, both of which may be associated with the recently described molecular subtypes of ES and MRT tumors (47). Additional synthetic lethal strategies may also be developed based on the immunologic effects of EZH2 inhibitors, particularly as combined with epigenetic and synthetic lethal therapies, both of which can promote tumor immunogenicity. In all, this study develops a paradigm for rational epigenetic combination therapy, including candidate prognostic biomarkers, all of which should be incorporated into future clinical trials for patients.

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Conflict of Interest

The authors have no competing financial interests. AK is a consultant for Novartis, Rgenta, and Blueprint.

Methods

EZH2 mutant plasmids

The *EZH2*^{Y666N} mutation detected in the clinical trial patient refers to amino acid numbering in isoform 2 of the protein. For consistency of nomenclature, all engineered mutations use numbering referring to isoform 2 (Uniprot ID: Q15190-2), although isoform 1 was expressed in cells for this study. Plasmids containing wild-type EZH2 (*EZH2*^{WT}) and catalytically inactive triple mutant (F672I, H694A, R732K, referred to as *EZH2*^{CatMut}) plasmids were kindly provided by Alejandro Gutierrez in the doxycycline-inducible pINDUCER20 vector (75). The plasmids contain human *EZH2* tagged N-terminally with a FLAG-Avi tag.

The Y666N mutation was engineered in both the *EZH2*^{WT} and *EZH2*^{CatMut} plasmids to yield *EZH2*^{Y666N} and what we termed *EZH2*^{QuadMut}. The mutation was introduced by site-directed mutagenesis using the QuikChange Lightning Kit (Agilent) per manufacturer's instructions (mutagenesis primers: 5'-GCAAAGTGTACGACAAGAACATGTGCAGCTTTCTG-3' and 5'-CAGAAAGCTGCACATGTTCTTGTCGTACACTTTGC-3') to engineer a TAC to AAC codon change. After mutagenesis, PCR products were transformed into Stbl3 *E. coli* cells and expanded at 30°C. Correct plasmid sequences were confirmed by Sanger sequencing (Eton). The sequencing primers used are:

F1: 5'-GGACAGCAGAGATCCAGTTTG-3'

R1: 5'-GGTCCGTTCCAGGATCTTCT-3'

F2: 5'-TCCAGTGTGGTGGAATTCTG-3'

R2: 5'-TATCGCTGGGGAACTTTCTG-3'

F3: 5'-TGCTGCACAACATCCCTTAC-3'

R3: 5'-TGCTGGTTTCGTCCTTCTTT-3'

F4: 5'-CCTACAAGCGGAAGAACACC-3'

R4: 5'-GTTCTTGCTGTCCCAGTGGT-3'

F5: 5'-CTGAAGAAGGATGGCAGCTC-3'

R5: 5'-CTTGGGTGGGTTACTCCAGA-3'

RB1 Knockout

G401 cells with *RB1* mutations were engineered by Synthego. Briefly, a single guide RNA targeting exon 2 of *RB1* was used (AGAGAGAGCUUGGUUAACUU). Ribonucleprotein containing spCas9 and sgRNA was transfected into G401 cells by electroporation. The target

site was then PCR-amplified and Sanger sequenced to ensure homozygous indels (PCR and sequencing primers: Forward- CACTGTGTGGTATCCTTATTTTGGA, Reverse- AGGTAAATTTCCTCTGGGTAATGGA, with the forward primer used for sequencing). The cells were then single-cell cloned and re-verified by Sanger sequencing. Loss of the RB1 protein was confirmed by Western blot.

Cell cycle analysis

G401 cells were plated on Day 0 and treated for 10 days with 1 µm tazemetostat or equivalent volume of DMSO, with drug and media replaced on Days 4 and 7. On Day 11, cells were pulsed with EdU for 1 hour. Cells were then harvested and processed for flow cytometry using the manufacturer's protocol (Click-iT, Invitrogen). Briefly, cells were washed with PBS with 1% BSA, permeabilized, and incubated with AlexaFluor647 for 30 minutes. DNA content was measured using propidium iodide (0.05 µg/µL). Cells were analyzed on a CytoFLEX LX (Beckman Coulter).

Patient tumor samples

Patient tumor and matched normal blood samples were obtained from patients at Memorial Sloan Kettering Cancer Center (MSKCC) enrolled in the TAZ clinical trial (13). All patients provided informed consent for this study under the Institutional Review Board approved research protocol 12-245. Patient tumors were classified into "Response" or "Progression" groups based on RECIST 1.1 criteria (76). "Response" included tumors exhibiting a complete response, partial response, or stable disease. All other tumors were classified under the "Progression" group. The complete list of tumor samples used and corresponding clinical data may be found in Supplementary Table 1. This cohort includes both tumor samples that underwent targeted sequencing with MSK-IMPACT (16) as part of their clinical care at MSKCC as well as archived tumors that were analyzed for this study. For genomic analysis, DNA was extracted from either flash frozen tumor samples or formalin-fixed, paraffin-embedded (FFPE) blocks or slides and samples were processed using the IMPACT468 or IMPACT505 panels depending on the time of their sequencing (16). The detected mutations and copy number alterations were obtained from cBioPortal (77, 78) and can be found in Supplementary Table 2. Oncoprints were generated using Oncoprinter (cBioPortal).

For transcriptomic analysis, archived frozen tumor samples were weighed and up to 20-30mg were homogenized in RLT buffer, followed by extraction using the AllPrep DNA/RNA Mini Kit

(QIAGEN catalog 80204) according to the manufacturer's instructions. RNA was eluted in 13 µL nuclease-free water. After RiboGreen quantification and quality control by Agilent BioAnalyzer, 1 µg of total RNA with DV200 percentages varying from 78% to 100% underwent ribosomal depletion and library preparation using the TruSeq Stranded Total RNA LT Kit (Illumina catalog RS-122-1202) according to instructions provided by the manufacturer with 8 cycles of PCR. Samples were barcoded and sequenced using NovaSeq 6000 in a PE150 mode, with the NovaSeq 6000 S4 Reagent Kit (Illumina). On average, 84 million paired reads were generated per sample and 70% of the data mapped to mRNA.

Targeted sequencing of cell lines

To assess for the presence of somatic mutations in MRT and ES cell lines, DNA was extracted using the PureLink Genomic DNA Minikit (Invitrogen) and processed using the IMPACT505 panel as above. Due to the lack of matched normal tissue for cell lines, copy number alterations were detected using a custom algorithm using circular binary segmentation (79) implemented by the MSK Bioinformatics core. Code is available on github at: https://github.com/kentsisresearchgroup/seqCNA_tazemetostat_resistance.

Additional methods may be found in the Supplementary Methods and Figures file.

Data Availability

RNA-seq data from G401 cells can be found at the Gene Expression Omnibus (GEO) repository, accession number GSE213845. RNA-seq data from patient tumor samples has been deposited to the Database of Genotypes and Phenotypes (dbGaP), accession number phs003188.v1.p1. Additional supplementary data files and computational analysis scripts are available at Zenodo (10.5281/zenodo.7595037).

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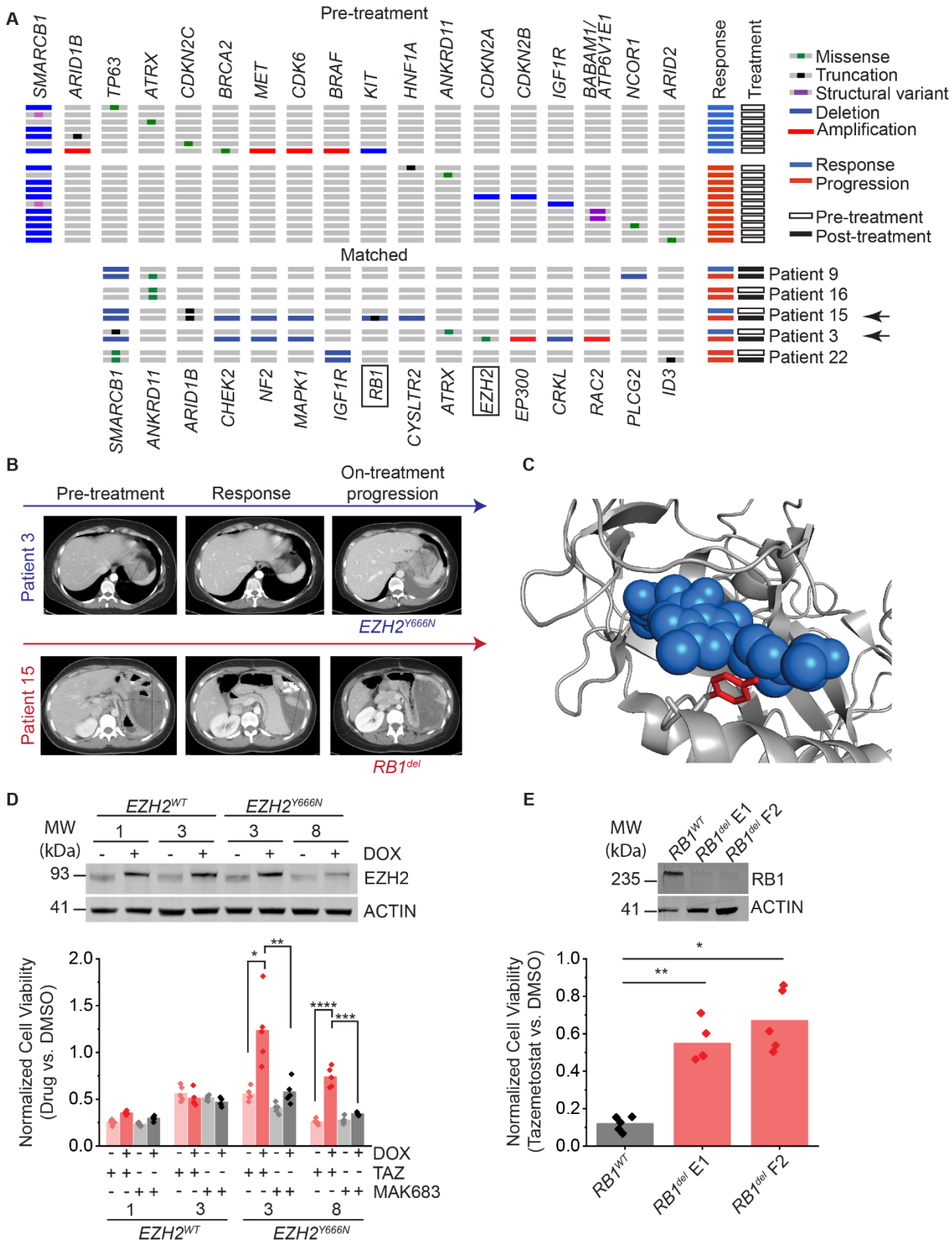

Figure 1: Patient tumor sequencing reveals diverse tazemetostat resistance mutations. (**A**) Abridged oncoprint of selected genes from MSK-IMPACT sequencing on patient tumor

samples. Top panel: Tumor samples prior to TAZ treatment. Bottom panel: Matched samples pre- and post-TAZ or pre- and post-acquisition of resistance. (**B**) Pre- and post- treatment CT imaging of the indicated patient tumors which acquired *EZH2* and *RB1* mutations. (**C**) Atomic molecular model of the chimeric *Homo sapiens/Anolis carolinensis* EZH2 bound to pyridone-based EZH2 inhibitor I (blue), PDB: 5IJ7. Y666 is highlighted in red. (**D**) Top panel: Doxycycline-inducible *EZH2* is expressed in single-cell G401 clones at near-physiological levels after 3 days treatment with doxycycline at 1 µg/mL. Numbers indicate clone ID. Bottom panel: Cell viability measured by CellTiter-Glo after 14 days of treatment with the indicated drug at 10 µM or equivalent volume of DMSO. n=5 biological replicates per condition. *p = 3.5E-3, **p = 5.2E-3, ***p = 3.8E-5, ****p = 1.1E-5 by two-sided Student's t-test. (**E**) Top panel: *RB1* knockout in two G401 clones (E1 and F2). Bottom panel: Cell viability measured by CellTiter-Glo after treatment with 10 µM tazemetostat or DMSO for 14 days. n=5 biological replicates per condition. *p = 2.8E-5, **p = 9.0E-5 by two-sided Student's t-test.

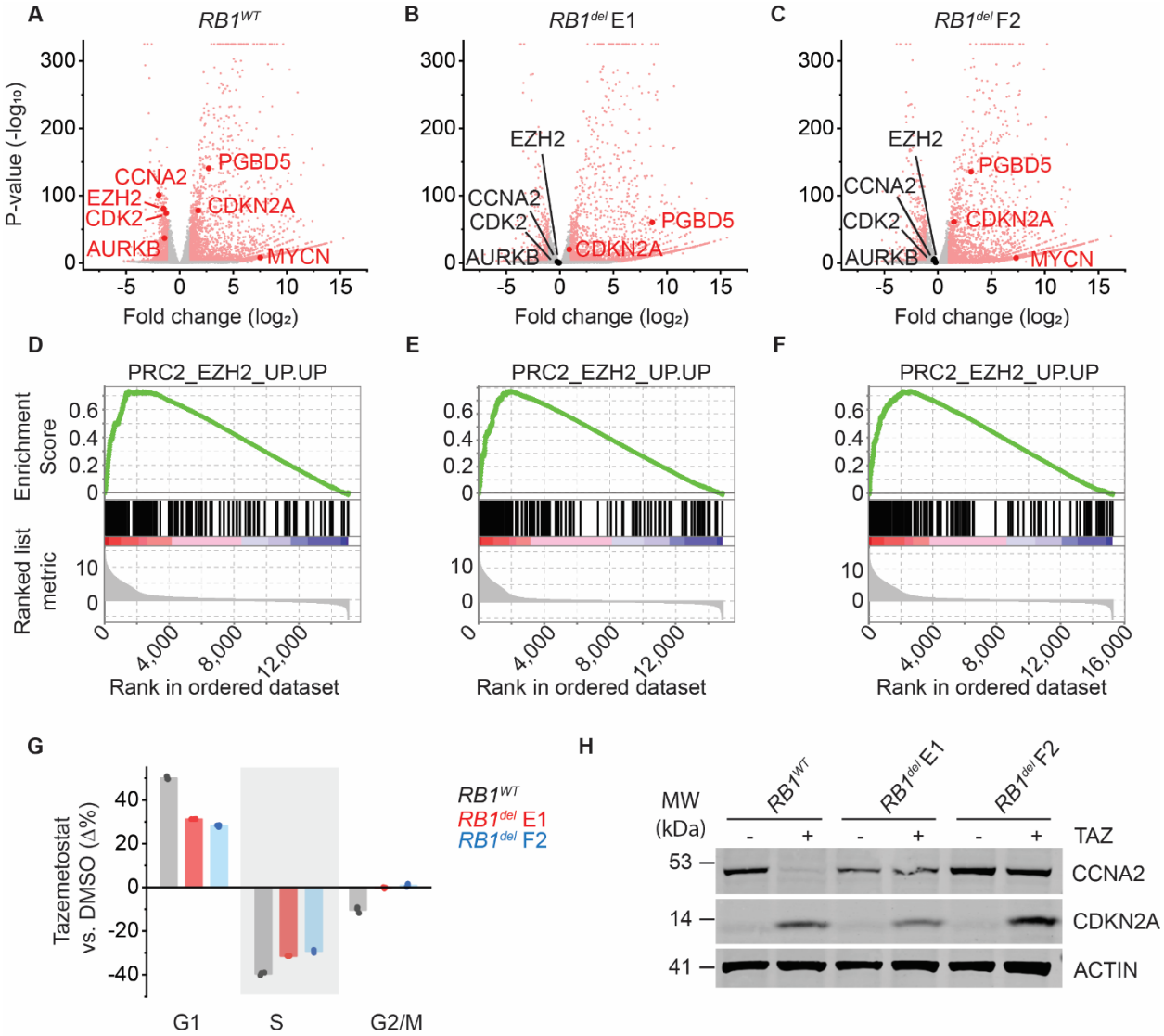

Figure 2: *RB1* loss allows escape from cell cycle arrest despite effective EZH2 inhibition. (**A-C**) Volcano plots of RNA-seq data from TAZ-treated G401 *RB1*^{WT} (**A**), *RB1*^{del} *E1* (**B**), and *RB1*^{del} *F2* (**C**). Plots show gene expression changes of cells treated with 10 µM TAZ versus equivalent volume of DMSO for 11 days. n=3 biological replicates per condition. Dots in red show genes with expression changes of $\log_2$(fold change) > ±1 and *p*-value < 0.01. (**D-F**) GSEA plots showing the PRC2_EZH2_UP.UP gene set for G401 *RB1*^{WT} (**D**), *RB1*^{del} *E1* (**E**), and *RB1*^{del} *F2* (**F**) cells treated in A-C. (**G**) Cell cycle analysis of the indicated G401 clone. Plot shows triplicate measurements of cells treated with 1 µM TAZ versus equivalent volume of DMSO for 11 days. Y-axis shows the percent difference of the TAZ-treated cells in each cell cycle phase compared to DMSO-treated cells. (**H**) Western blot of the indicated G401 cell clones treated with 10 µM TAZ versus equivalent volume of DMSO for 11 days. Actin serves as loading control.

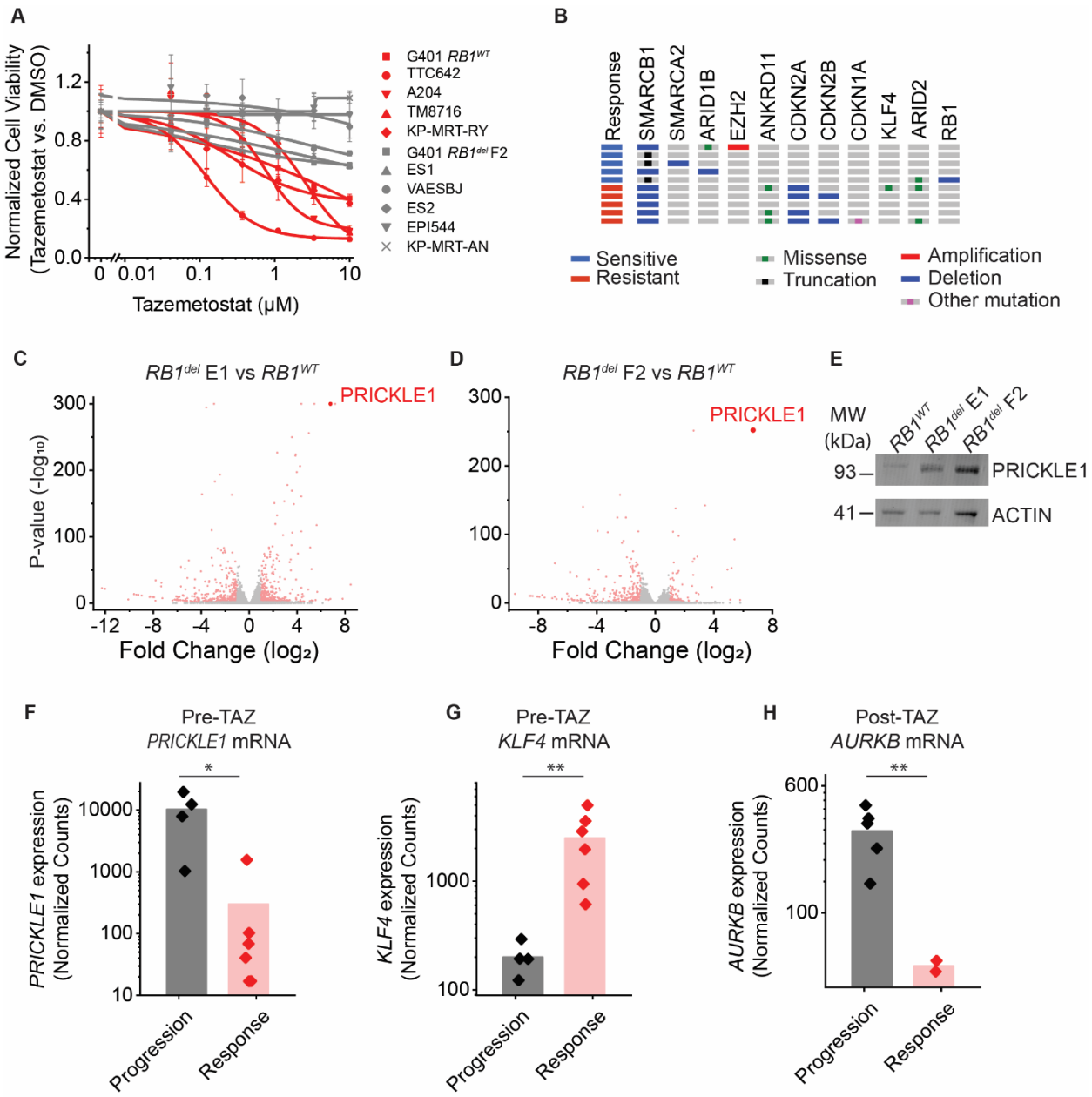

Figure 3: An intact RB1/E2F axis is a key requirement for response to TAZ: (**A**) Dose-response curves of a panel of MRT and ES cell lines treated with TAZ for 13 days. Curves in red correspond to TAZ-responsive cell lines, grey to TAZ-resistant cell lines. (**B**) Abridged oncoprint of MRT and ES cell lines. Genes included here are BAF subunits and CDK4/6/RB1/E2F axis genes. Full list of mutations is listed in Supplementary Table 4. (**C-D**) Volcano plots of RNA-seq data comparing DMSO-treated G401 *RB1*^{del} E1 (**C**) and F2 (**D**) with *RB1*^{WT} cells. (**E**) Western blot for PRICKLE1 in untreated G401 cells. (**F-G**) Read counts for *PRICKLE1* (**F**) and *KLF4* (**G**) showing counts normalized by DESeq2 for patient tumor samples collected prior to TAZ treatment. (**H**) Read counts for *AURKB* for patient tumor samples collected after TAZ treatment. *p=0.013, **p=0.03.

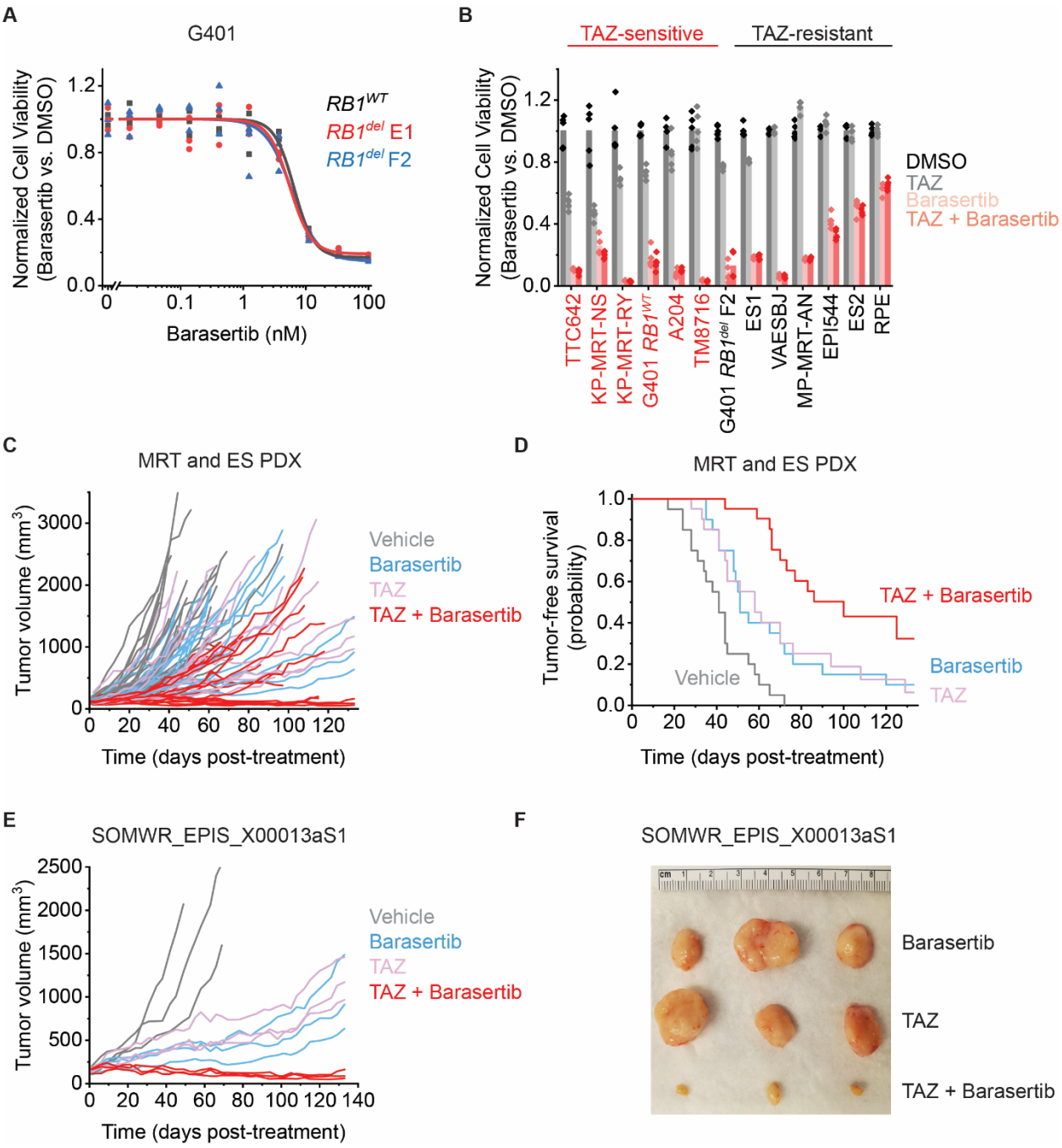

Figure 4: Cell cycle bypass combination strategy using AURKB inhibition overcomes TAZ resistance and improves response: (**A**) G401 cells treated with barasertib for 6 days (**A**). (**B**) Panel of MRT and ES cell lines ordered left to right by decreasing response to TAZ monotherapy. Cells were treated with the indicated monotherapy or combination for 11 days. Drug concentrations used were: TAZ: 200 nM, barasertib: 8 nM. (**C**) Tumor growth curves showing volumes calculated from caliper measurements for 5 mouse PDXs treated with the indicated drug regimen. n = 20 mice for vehicle and barasertib-treated groups, n = 21 for TAZ and TAZ + barasertib-treated groups. Vardi *U*-test *p* = 4.0E-4 and 2.0E-4 for combination vs. barasertib or TAZ, respectively. (**D**) Kaplan-Meier curves showing tumor-free survival (defined

as tumor volume ≤ 1,000 mm^3) for the PDXs in panel C. Mean survival is 65 days (95% CI: 51-78 days) for barasertib, 67 days (95% CI: 53-81 days) for TAZ, 98 days (95% CI: 84-112 days) for the combination. Log-rank test p = 3.3E-3 and 5.8E-3 for combination vs. barasertib or TAZ, respectively. (**E**) Tumor growth curves for the subset of mouse tumors in panel C from the SOMWR_EPIS_X00013aS1 PDX model. n = 3 mice per treatment group. Vardi *U*-test p = 0.10 and 9.4E-2 for combination vs. barasertib or TAZ, respectively. (**F**) Tumors from panel E harvested on Day 135 of treatment.

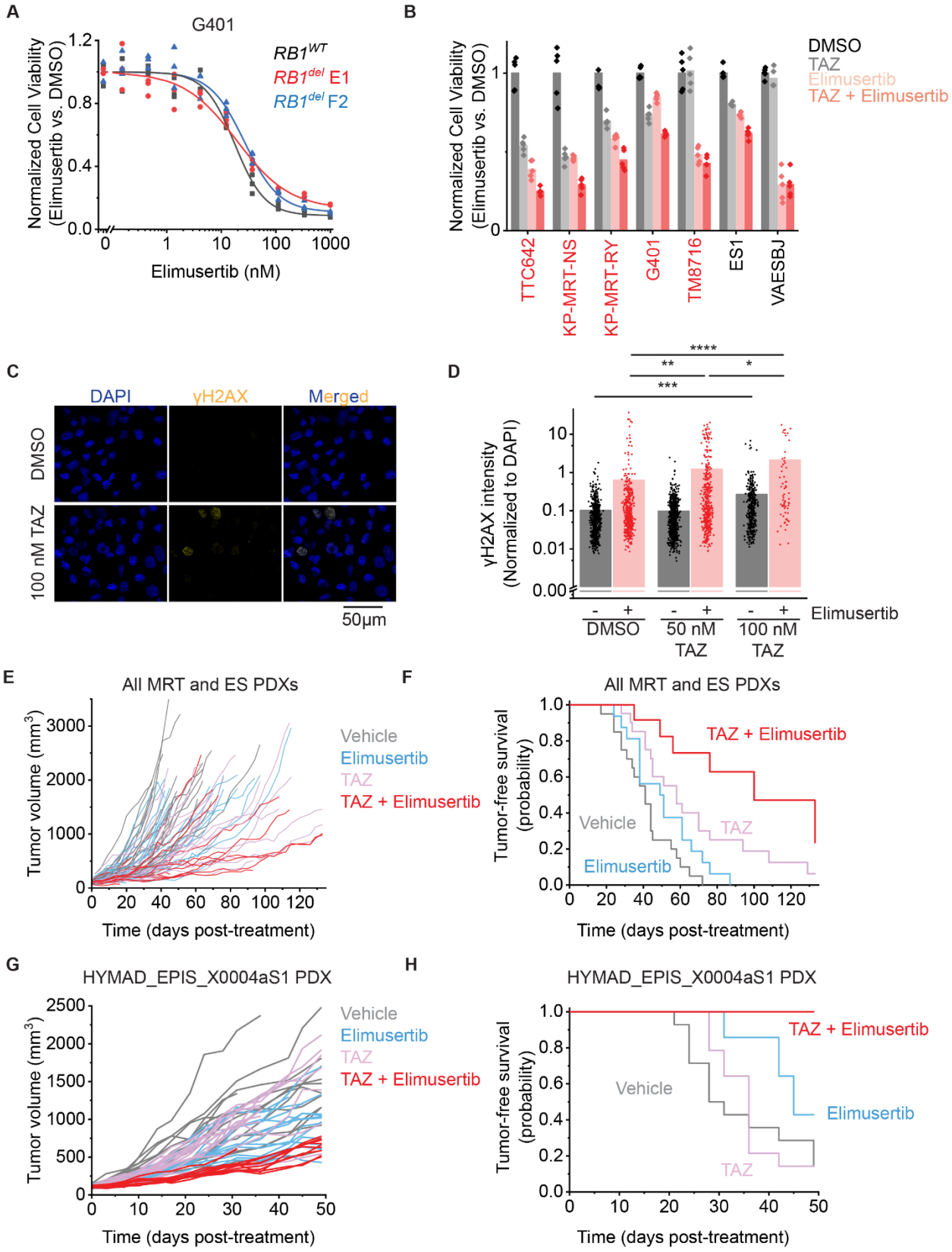

Figure 5: Synthetic lethal combination strategy using ATR inhibition overcomes TAZ resistance and improves response: (**A, C**) G401 cells treated with elimusertib for 4 days (**C**).

(**B**) Panel of MRT and ES cell lines ordered left to right by decreasing response to TAZ monotherapy. Cells were treated with the indicated monotherapy or combination for 11 days. Drug concentrations used were: TAZ: 200 nM, elimusertib: 8 nM. We selected an elimusertib dose below its monotherapy IC_{50} (for G401 cells) in order to visualize any additive effects upon combination with TAZ. (**C**) Representative images of G401 cells treated with the indicated treatment for 7 days. (**D**) Quantification of γH2AX fluorescence relative to DAPI fluorescence. *p = 0.042, **p = 5.1E-3, ***p = 1.7E-8, ****p = 6.5E-4 by two-sided Student's t-test. n = 548 nuclei for DMSO, 432 for elimusertib, 696 for 50 nM TAZ, 401 for 50 nM TAZ + elimusertib, 373 for 100 nM TAZ, 63 for 100 nM TAZ + elimusertib. (**E**) Tumor growth curves for 5 mouse PDXs treated with the indicated drug regimen. n = 20 mice for vehicle and elimusertib-treated groups, n = 21 for TAZ and TAZ + elimusertib-treated groups. Vardi *U*-test p = 3.2E-2 and 0.23 for combination vs. elimusertib or TAZ, respectively. (**F**) Kaplan-Meier curves showing tumor-free survival (defined as tumor volume ≤ 1,000 mm^3) for the PDXs in panel C. Mean survival is 51 days (95% CI: 42-60 days) for elimusertib, 67 days (95% CI: 53-81 days) for TAZ to 99 days (95% CI: 74-123 days) for the combination. Log-rank test p = 5.8E-4 and 3.9E-2 for combination versus elimusertib or TAZ, respectively. (**G**) Tumor growth curves for the HYMAD_EPIS_X0004aS1 PDX model treated with the indicated drug regimen. Vardi *U*-test p = 2.0E-4 for combination versus elimusertib or TAZ. n = 14 mice per treatment group. (**H**) Kaplan-Meier curves showing tumor-free survival (defined as tumor volume ≤ 1,000 mm^3) for the PDXs in panel E. Log-rank test p = 6.2E-3 and 6.3E-5 for combination versus elimusertib or TAZ, respectively.

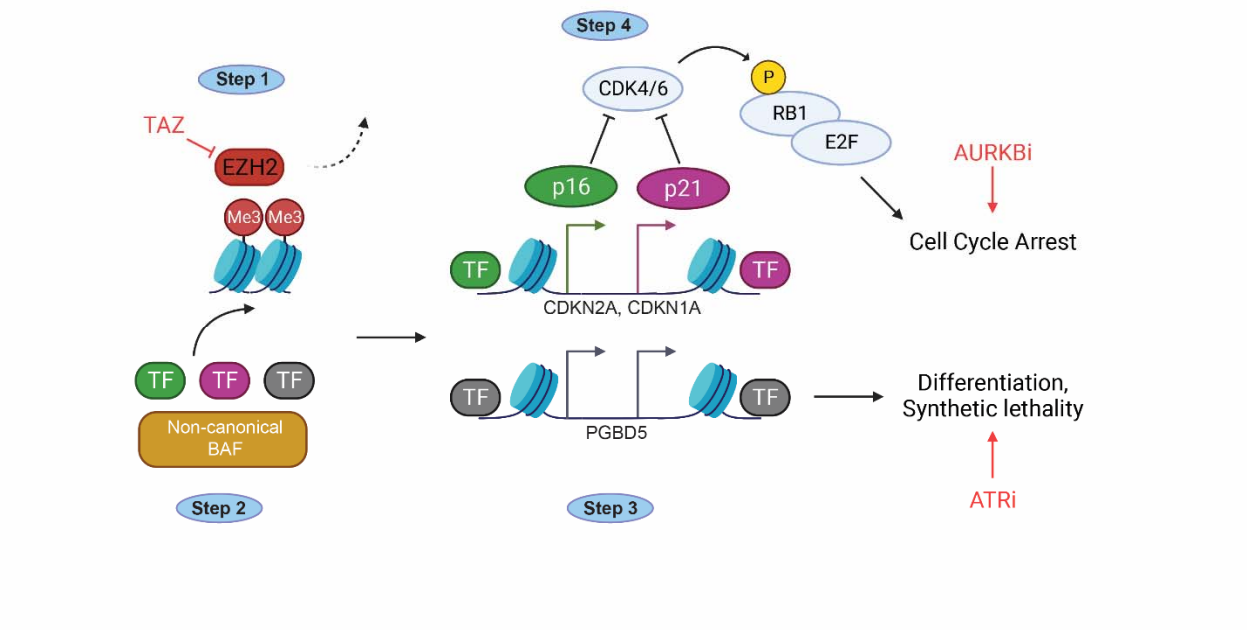

Figure 6: Mechanistic schematic for the response of BAF-deficient tumors to effective EZH2 therapy: Step 1: TAZ inhibits histone methylation activity of PRC2. **Step 2:** Activating chromatin-bound complexes, such as ncBAF bind to tumor suppressor loci. **Step 3:** Tumor

suppressor loci are activated by their transcription factors and their coactivators. **Step 4:** Tumor suppressors inhibit cell cycle progression through their downstream effectors, such as RB1/E2F.