A cytoskeletal function for PBRM1 reading methylated microtubules

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Epigenetic effectors “read” marks “written” on chromatin to regulate function and fidelity of the genome. Here, we show that this coordinated read-write activity of the epigenetic machinery extends to the cytoskeleton, with PBRM1 in the PBAF chromatin remodeling complex reading microtubule methyl marks written by the SETD2 histone methyltransferase. PBRM1 binds SETD2 methyl marks via BAH domains, recruiting PBAF components to the mitotic spindle. This read-write activity was required for normal mitosis: Loss of SETD2 methylation or pathogenic BAH domain mutations disrupt PBRM1 microtubule binding and PBAF recruitment and cause genomic instability. These data reveal PBRM1 functions beyond chromatin remodeling with domains that allow it to integrate chromatin and cytoskeletal activity via its acetyl-binding BD and methyl-binding BAH domains, respectively. Conserved coordinated activity of the epigenomic machinery on the cytoskeleton opens a previously unknown window into how chromatin remodeler defects can drive disease via both epigenetic and cytoskeletal dysfunction.

INTRODUCTION

The epigenome consists of DNA and histone modifications that control the structure, function, and fidelity of the genome. The epigenetic machinery is responsible for establishing, modifying, and maintaining the epigenome consists of proteins acting in an integrated manner to recognize (“read”), add (“write”), or remove (“erase”) epigenetic marks such as methylation. The functional relationships between specific epigenetic marks and their cognate readers, writers, and erasers are highly coordinated and conserved across eukaryotic evolution to precisely regulate chromatin conformation and gene expression and facilitate DNA repair.

The epigenetic reader PBRM1 (polybromo-1), also known as PB1 or BAF180, is a defining subunit of the PBAF (polybromo BRG1-associated factor) chromatin remodeler complex. PBAF belongs to the SWI/SNF family in mammalian cells (1), with homology to the RSC (remodeling the structure of chromatin) complex in yeast (2). PBRM1, along with two other subunits, BRD7 and ARID2, distinguishes PBAF from other SWI/SNF complexes, termed BAF and ncBAF (3). PBRM1 is a large protein containing six BD (acetyl-binding bromo) domains with canonical activity binding acetylated histones as part of a nuclear PBAF chromatin remodeling complex (4). Defects in PBAF and other SWI/SNF adenosine triphosphate (ATP)-dependent chromatin remodeling complexes have emerged as important drivers of cancer (5, 6) and neurodevelopmental diseases such as autism spectrum disorder (ASD) (7).

The histone methyltransferase set domain containing 2 (SETD2) is an epigenetic writer that adds a trimethyl mark to lysine-36 on the tail of histone H3 (8). This H3K36me3 epigenetic mark participates in transcriptional elongation and alternative splicing of RNA and DNA damage repair (8–10). Similar to PBRM1, loss of SETD2 is frequently observed in several cancer types (11, 12) and ASD (13). Recently, we reported that, in addition to histones, SETD2 also methylates lysine-40 of α-tubulin (α-TubK40me3) on microtubules (14, 15) and lysine-68 (ActK68me3) on actin (16), which localizes to spindle microtubules during mitosis and the midbody during cytokinesis, and the leading edge of cells, respectively. Loss of SETD2 and α-TubK40me3 causes genomic instability and defects such as multipolar spindle formation, chromosomal bridges at cytokinesis, micronuclei, polyploidy, and polynuclease, a phenotype specifically linked SETD2 activity as a tubulin methyltransferase (14, 15).

RESULTS

PBRM1 binds α-tubulin and colocalizes with the SETD2 methyl mark on spindle microtubules during mitosis

As shown in Fig. 1A and fig. S1A, we found that while PBRM1 localized primarily to the nucleus during interphase, in mitotic cells PBRM1 localization was highly enriched at the poles of the mitotic spindle. This localization pattern was observed using both a green fluorescent protein (GFP)-tagged PBRM1 fusion protein or an antibody directed against endogenous PBRM1 in several cell types.
Fig. 1. PBRM1 binds α-tubulin and colocalizes with the SETD2 methyl mark on spindle microtubules during mitosis. (A) Representative images of HEK293T cells ectopically expressing GFP-PBRM1 (green) and stained with antibodies specific for the SETD2 methyl mark (α-TubK40me3 red), α-tubulin (far red/purple), and 4′,6-diamidino-2-phenylindole (DAPI) to visualize chromosomes (blue) showing PBRM1 colocalization with α-TubK40me3 and α-tubulin at mitotic spindle and spindle pole (lower panels). (B) To demonstrate the colocalization of PBRM1 (green) and α-TubK40me3 (red), line profiles were obtained between the two spindle poles using deconvolution microscope NIS elements software, which showed that green and red signals are aligned at spindle pole and PBRM1 and α-TubK40me3 are colocalized. Scale bars, 5 µm (n = 3 biological replicates). (C) Representative images from deconvolution microscopy showing localization of PBRM1 to the mitotic spindle in SETD2+/+ and SETD2−/− 786-O cells during mitosis stained for PBRM1 (purple) and α-tubulin (red) and DAPI to visualize chromosomes (blue). Scale bars, 5 µm (n = 3 biological replicates). (D) Representative images of line intensity profiles and intensity measurement of PBRM1 on mitotic spindle in SETD2+/+ and SETD2−/− 786-O cells using deconvolution microscope NIS elements software. Scale bars, 5 µm (n = 3). (E) Quantification of intensity at peak spindle pole localization of PBRM1 from (D) in SETD2+/+ and SETD2−/− 786-O cells. Data are represented as means ± SEM. P value was determined by t test (n = 45 mitotic cells per condition). (F) Immunoblot (IB) analysis showing coimmunoprecipitation (IP) of endogenous α-tubulin and PBRM1 and respective input lysates from HEK293T cells and PBRM1 CRISPR-KO HEK293T cells. COX5A serves as a negative control and shows the specificity of the interaction of PBRM1 with α-tubulin. (G) IB analysis showing coimmunoprecipitation of endogenous PBRM1 and ectopically expressed α-tubulin and respective input lysates from HEK293T cells. Representative blots (n = 3).
including human kidney epithelial [human kidney cell (HKC)], 786-O [clear cell renal cell carcinoma (ccRCC)], and human embryonic kidney (HEK) 293T (fig. S1, A to E). Focal immunoreactivity at spindle poles was specific for PBRM1, as this pattern of localization was seen in PBRM1-proficient but not PBRM1-deficient cells (fig. S1B). To further confirm the spindle localization of PBRM1 during mitosis, we performed live-cell imaging of GFP-PBRM1 in HEK293T cells, which showed PBRM1 localizing to the mitotic spindle, with most intense localization at the spindle pole (movie S1).

PBRM1 binding to spindle microtubules colocalized with the α-TubK40me3 mark made by the SETD2 methyltransferase (14, 15), evident from colocalization of antibodies directed against the α-TubK40me3 mark and GFP-tagged PBRM1, and confirmed by overlay of the peak signal intensity of line profiles through the mitotic spindle of these images (Fig. 1, A and B). Moreover, focal localization of PBRM1 to spindle poles was significantly reduced in SETD2-null cells deficient for α-TubK40me3 (Fig. 1, C to E, and fig. S2, A to C). Loss of PBRM1 from spindle poles was not due to loss of PBRM1, as SETD2-deficient 786-O cells retained PBRM1 expression (fig. S2D).

To establish the specificity of the observed interaction of PBRM1 with microtubules, we performed microtubule co-sedimentation assays using lysates prepared from parental or PBRM1 CRISPR-KO (knockout) HEK293T cells using COX5A (no known microtubule binding) as a negative control and MAP4 and p150Glued (known microtubule-associated proteins) as positive controls, respectively. PBRM1, MAP4, and p150Glued all co-sedimented with taxol-stabilized microtubules (fig. S2D).

PBRM1 recognizes the SETD2 methyl mark on α-tubulin via its BAH binding domains

PBRM1 has six bromodomains and two BAH domains (Fig. 2A), although the target for its predicted methyl-binding BAH domains is unknown. PBRM1 recognizes the histone H3 lysine-14 acetyl (H3K14ac) mark on chromatin via its acetyl-binding BD domains, particularly BD 2 and 4 (19–21). Because lysine-40 of α-tubulin, the target for SETD2 methylation, can be either acetylated (α-TubK40ac) (22) or methylated (α-TubK40me3) (15), we asked which domains of PBRM1 were responsible for microtubule binding. Pull-down assays using glutathione S-transferase (GST) fusion proteins for the six BD and two BAH domains of PBRM1 revealed both BAH domains, but none of the six acetyl-binding bromodomains recognized α-tubulin, with BAH2 showing the stronger interaction (Fig. 2B). These data suggested that PBRM1 binds to methylated, rather than acetylated, α-tubulin. To test this, we expressed enhanced GFP-tagged wild type, K40Q (acetylation mimic), and K40R (methyl and acetyl deficient) mutant α-tubulin in HEK293T cells and found that PBRM1 association with α-tubulin was markedly reduced by both the K40R and K40Q mutations (Fig. 2C). To confirm that PBRM1 specifically recognized the α-TubK40me3 SETD2 methyl mark, we synthesized α-tubulin tetrapeptides with unmodified, acetylated, or trimethylated lysine-40 and performed peptide pull-down assays, which confirmed that PBRM1 bound to trimethylated, but not acetylated or unmodified, α-tubulin K40 peptides (Fig. 2D and fig. S3D). Last, and consistent with loss of PBRM1 spindle localization in SETD2-deficient cells, coimmunoprecipitation of PBRM1 and α-tubulin was reduced in SETD2-null methyl-deficient cells (Fig. 2E). However, binding was not completely abrogated in the setting of SETD2 loss (see dark exposure in Fig. 2E), suggesting residual binding due to recognition of methyl marks made by other methyltransferases on α-tubulin, or binding to K40 mono- or di-methyl marks that persist in the absence of SETD2 K40 trimethylation. Thus, PBRM1 is a microtubule-binding protein that recognizes the SETD2 α-TubK40me3 mark on microtubules via its BAH methyl-binding domains.

PBRM1 recruits SWI/SNF (PBAF) subunits to SETD2-methylated microtubules

SWI/SNF components typically coexist as a complex in cells, and the presence of one suggests the presence of others (23). We thus asked whether other components of the PBAF complex localize with PBRM1 to mitotic microtubules. Both PBRM1 and the adenosine triphosphatase (ATPase) subunit of PBAF (SMARCA4/BAF180) could be detected in the cytoplasmic fraction of cells by Western analysis (fig. S4A). This allowed us to perform mass spectrometry from this subcellular compartment to identify PBAF components that could associate with PBRM1 in the absence of chromatin. Following immunoprecipitation of PBRM1 from the cytoplasmic fraction of HEK293T cells, we confirmed by mass spectrometry that PBRM1 exists in a chromatin-independent complex with the ATPase BRG1, and several other PBAF components including ARID2 (BAF200), BRD7, SMARCB1 (BAF47), and SMARCC1 (BAF155) (fig. S4, B and C, and table S1).

Consistent with PBRM1 binding to microtubules as part of a PBAF complex, in addition to the BRG1 ATPase, the PBAF component ARID2 could also be coimmunoprecipitated with α-tubulin (Fig. 3A), and both BRG1 and ARID2 localized to the spindle pole during mitosis (Fig. 3B). Knockdown of PBRM1 abrogated spindle localization of both BRG1 and ARID2 (Fig. 3B), indicating that the interaction of these PBAF components with microtubules was PBRM1 dependent. This dependence on PBRM1 for PBAF interaction with α-tubulin was further confirmed after PBRM1 knockdown by loss of the ATPase BRG1 binding to α-tubulin, which was rescued by reexpression of PBRM1 (Fig. 3C). Using GFP-tagged BRG1, in HEK293T and HKC cells, we observed that as with PBRM1, BRG1 was predominantly nuclear in interphase cells but localized to spindle poles during mitosis (fig. S5A), where it colocalized with PBRM1 (fig. S5B).

Specificity for this interaction with methylated α-tubulin was shown, as BRG1 and ARID2 coimmunoprecipitated along with PBRM1 using an α-tubulin peptide with the SETD2 K40me3 mark, but not unmethylated or acetylated α-tubulin peptides (Fig. 3D). As seen with loss of the methyl reader PBRM1, loss of the SETD2 writer and the α-TubK40me3 mark resulted in decreased BRG1 binding to α-tubulin (Fig. 3E) and significantly reduced focal localization of
BRG1 at the spindle pole (fig. S5, C to E). Together, these findings demonstrate that PBRM1 functions as a reader of the SETD2 methyl mark that recruits key components of a PBAF complex, including the ATPase BRG1 and ARID2, to focally localize PBAF to the poles of spindle microtubules.

**Oncogenic mutations in PBRM1 BAH domains impair recognition of the SETD2 methyl mark, binding to spindle microtubules and genomic stability**

The BAH domains of PBRM1 are frequent sites for oncogenic alterations in cancer (24, 25). This led us to ask whether pathogenic

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**Fig. 2. PBRM1 interacts with α-tubulin via its BAH methyl-binding domain.** (A) Schematic of PBRM1 protein showing its various domains including six bromodomains, two BAH domains, and an HMG domain. (B) GST pull-down assay for α-tubulin using GST fusion proteins for each of six bromodomains (BD) and two BAH domains incubated with 1 μg of porcine brain microtubule protein and immunoblotted using α-tubulin antibody (upper panel). GST-PBRM1 BD and BAH domain construct expression was assessed using Coomassie blue staining (lower panel). Representative blots and gels (n = 3). (C) IB analysis following coimmunoprecipitation of PBRM1 and ectopically expressed wild-type (WT) or mutant α-tubulin (K40R) or acetylation mimic (K40Q) and respective input lysates from HEK293T cells. Representative blots (n = 3). (D) IB analysis following peptide pull-down using biotin-labeled K40 α-tubulin peptides that were unmodified (K40-UN), acetylated (K40-Ac), or trimethylated (K40-me3) using lysates from PBRM1-proficient (HEK293T) or PBRM1-deficient (RCC4) cells. Representative blots are shown (n = 3). (E) IB analysis showing coimmunoprecipitation of endogenous PBRM1 and α-tubulin, and their respective input lysates from SETD2+/+ and SETD2−/− 786-O cells. Residual signal detected with α-TubK40me3 may be due to cross-reactivity with dimethylated K40, other methyl marks on α-tubulin, or redundancy with other α-tubulin methyltransferases acting at K40. Representative blots (n = 3).
Fig. 3. PBRM1 recruits SWI/SNF (PBAF) subunits to methylated microtubules. (A) IB analysis showing coimmunoprecipitation of BRG1 and ARID2 with α-tubulin and their corresponding input lysates from HEK293T and HKC cells. Representative blot \( (n = 3) \). (B) Representative image using deconvolution microscopy of cells stained with antibodies directed against BRG1 (red, upper two panels) or ARID2 (red, lower two panels) and α-tubulin (green), DAPI to visualize chromosomes (blue). Representative images show BRG1 and ARID2 localization at the mitotic spindle and spindle pole in PBRM1-proficient HEK293T cells (upper panel) and loss of mitotic spindle localization in PBRM1-depleted HEK293T cells (lower panel). Scale bars, 5 μm \( (n = 3 \) biological replicates). (C) IB analysis showing coimmunoprecipitation of PBRM1 and α-tubulin, coimmunoprecipitation of BRG1 and α-tubulin, and their corresponding input lysates from PBRM1-proficient, PBRM1-depleted, and PBRM1-rescued HEK293T cells showing loss of interaction between BRG1 and α-tubulin with loss of PBRM1. Representative blots \( (n = 3) \). (D) IB analysis following peptide pull-down using biotin-labeled K40 α-tubulin peptides that were unmodified (K40-UN), acetylated (K40-Ac), or trimethylated (K40-me3) using lysates from PBRM1-proficient or PBRM1-depleted HEK293T cells showing interactions of BRG1, ARID2, and PBRM1 with K40 α-tubulin peptides is dependent on PBRM1. Representative blots \( (n = 3) \). (E) IB analysis showing dependency on SETD2 for coimmunoprecipitation of BRG1 with α-tubulin and their respective input lysates from SETD2\(^{+/+}\) and SETD2\(^{-/-}\) 786-O cells. Representative blots \( (n = 3) \).
mutations in the BAH domain could disrupt localization to microtubules and/or the ability of PBRM1 to maintain genomic stability. While truncating events that cause major disruptions in protein architecture are most common deleterious PBRM1 alterations, we were able to identify missense mutations in PBRM1 in The Cancer Genome Atlas (TCGA) database and chose three of these that occur in BAH1 and BAH2 domains for analysis: P1048R, T1202K, and C1233W (fig. S6A).

To determine the impact of these mutations on PBRM1 binding to α-tubulin, we first performed a structural analysis of the PBRM1 BAH1 and BAH2 domains. The structure of the human BAH1 domain was recently determined (26). This structure [Protein Data Bank (PDB): 6OXB] showed that P1048 in the human BAH1 is located in a hydrophobic groove (fig. 4A). Modeling of arginine in place of proline at this position showed that the arginine side chain, in addition to altering the hydrophobic nature of the groove, caused severe steric clashes with V1036 and F1085 (fig. 4A), suggesting that a P1048R mutation would cause a major structural rearrangement in the hydrophobic groove. Such a disruption in terms of both hydrophobicity and structure would be likely to abrogate or greatly reduce methyl binding (Fig. 4B). As the structure of the BAH2 domain is not yet determined, we performed homology modeling using SWISS-MODEL (27), which autonomously chose the human BAH1 as a reference structure to generate a structural model for BAH2. This structure-based alignment showed a 70% similarity between human BAH1 and BAH2 structures (fig. S6B) and revealed that the C1233W mutation in BAH2 would also cause severe steric clashes with V1235, C1163, and V1164 (Fig. 4, C and D). This predicts that the C1233W mutation would also reduce methyl binding. In contrast, mutation of the surface-exposed T1202 residue to lysine (K) can be easily accommodated, predicting that T1202K mutation would have little effect on PBRM1 interaction with α-tubulin (Fig. 4, E and F).

On the basis of these structural data, we generated GFP-PBRM1 expression constructs for the P1048R, T1202K, and C1233W BAH domain mutations. Ectopic expression of these mutant constructs in PBRM1-deficient PBRM1 CRISPR-KO HEK293T cells revealed the mutations predicted to disrupt hydrophobic pocket structure, P1048R (BAH1) and C1233W (BAH2), significantly decreased PBRM1 binding to α-tubulin (Fig. 5, A and B). In contrast, T1202K (BAH2) did not impair PBRM1 interaction with α-tubulin as determined by conventional communoprecipitation or using GFP-Trap affinity resin immunoprecipitation (Fig. 5, A and B). Furthermore, localization of P1048R and C1233W binding-deficient PBRM1 mutants at the mitotic spindle pole was significantly reduced when compared with either wild-type or tubulin binding–proficient T1202K mutant PBRM1 (Fig. 5, C and D, and fig. S6C).

The C-terminal region of PBRM1 is primarily involved in interacting with the PBAF complex (3), and an oncogenic Q1298* frameshift mutation abrogates PBRM1 incorporation into this complex (28). Although the structural studies above would not suggest that this is the case, it remained a formal possibility that mutations abrogating tubulin binding did so via disruption of PBRM1 tertiary structure. Therefore, we next asked whether the tubulin binding–deficient P1048R or C1233W mutants could still assemble a PBAF SWI/SNF complex. Comminoprecipitation studies with mutant P1048R or C1233W PBRM1 showed that these mutants were still able to complex with PBAF-specific subunits BRG1, ARID2, and SMARCC1 (fig. S7A). Mass spectrometry performed after BRG1 pull-down confirmed that the stoichiometry of PBAF component complexed PBRM1 was similar between mutant (P1048R) and wild-type PBRM1 (table S2). In addition, after normalization for pull-down efficiency, there was no significant difference between the amount of BRG1 or ARID2 immunoprecipitated with wild-type or α-tubulin binding–proficient T1202K mutant and the α-tubulin binding–deficient P1048R and C1233W PBRM1 mutants (fig. S7B).

In addition, we performed a transcriptional analysis of PBRM1-null cells rescued with either microtubule-binding proficient or microtubule-binding deficient PBRM1 constructs to determine whether PBAF complexes deficient for microtubule binding retained their activity on chromatin. RNA sequencing (RNA-seq) was used to define the shared transcriptional signature of microtubule binding–proficient PBRM1-null cell strains rescued with wild-type or T1202K PBRM1, which was then compared to the shared signature of the binding-deficient PBRM1-null cell strains rescued with P1048R or C1233W PBRM1. We found the transcriptomic profile of PBRM1-null cells rescued with α-tubulin binding–proficient (wild-type or T1202K mutant) PBRM1 was virtually identical to cells rescued with α-tubulin binding–deficient (P1048R or C1233W mutant) PBRM1, differing in the expression of only 15 (14 up- and 1 down-regulated) of 10,160 genes (fig. S7C). A previous RNA-seq study using ectopically expressed PBRM1 in a kidney cancer cell line (Caki2) found that PBAF regulates genes involved in cellular adhesion, carbohydrate metabolism, apoptotic processes, and response to hypoxia (29). Hallmark pathway analysis indicated that neither P1048R nor C1233W mutants affected the expression of genes in these pathways (fig. S7C), which, together with the RNA-seq data, indicate that oncogenic BAH1 domain mutations do not perturb PBRM1 chromatin function. Thus, while the P1048R and C1233W BAH domain mutations significantly disrupted PBRM1 binding to α-TubK40me3 and mitotic spindle localization, they preserved the structure of PBRM1 sufficiently so as not to disrupt PBRM1 association with other PBAF components or the transcriptional activity of PBAF complexes.

In yeast, the PBRM1 homolog RSC2 is essential for chromosome arm cohesion (30). PBRM1 was previously localized to the kinetochore in the absence of microtubules (31), and in a follow-on study, the induction of genomic instability observed upon loss of PBRM1 (32) was inferred to be due to loss of centromere cohesion. However, we did not see prominent localization of PBRM1, BRG1, or ARID2 at the centromere of mitotic chromosomes with our staining protocol (Figs. 1 to 3), prompting us to ask whether instead localization at the spindle pole was key to maintenance of genomic stability by PBRM1. In this regard, the binding-defective mutants provided tools to test the causal relationship between PBRM1 ability to read the SETD2 α-TubK40me3 mark and its function at the mitotic spindle.

CRISPR-KO of PBRM1 in HEK293T cells, a sensitive system for assessing perturbation of genomic stability, significantly increased the formation of lagging chromosomes, chromosome bridges, multipolar spindles (Fig. 6, A to D), and formation of micronuclei (fig. S8, A and B). Genomic instability (micronuclei) was also induced by small interfering RNA (siRNA) knockdown of PBRM1 (fig. S8C). This loss of the PBRM1 reader phenocopied the genomic instability previously reported for SETD2-null cells that had lost the α-TubK40me3 mark (14, 15). Reexpression of α-tubulin binding–proficient PBRM1 (wild-type or T1202K mutant) rescued this genomic
instability, whereas neither tubulin binding–deficient P1048R nor C1233W PBRM1 mutants were able to do so (Fig. 6, B and D, and fig. S8, A and B). Collectively, these data reveal that oncogenic BAH mutations in PBRM1, which preserve PBAF complex formation and transcriptional integrity, nevertheless cause functional defects in the ability of PBRM1 to recognize the SETD2 methyl mark, bind α-tubulin, and recruit PBAF complexes to spindle microtubules during mitosis to maintain genomic stability.

DISCUSSION
These studies identify PBRM1 as the first methyl reader for the α-TubK40me3 mark on microtubules. Recognition of the SETD2 α-TubK40me3 mark and recruitment of PBAF components to microtubules by PBRM1 reveals a coordinated functional relationship between an epigenetic writer and an epigenetic reader acting on the cytoskeleton. Although PBRM1 has both acetyl-binding BD and methyl-binding BAH domains, and microtubules can be acetylated or methylated at lysine-40, our data show that PBRM1 BAH domains specifically recognize the α-TubK40me3 SETD2 methyl mark. Thus, the chromatin and cytoskeletal functions of PBRM1 use distinct protein domains, allowing integration and coordination, rather than competition, for these activities in the cell (Fig. 7).

An intriguing aspect of microtubule biology is the luminal positioning of K40, target for both the α-TubK40me3 set by SETD2 and the α-TubK40ac acetyl mark made by α-tubulin acetyltransferase (ATAT-1). Luminal positioning of acetyl and methyl marks raises the question as to how writers, such as ATAT-1 and
Fig. 5. Oncogenic mutations in PBRM1 BAH domain disrupt interaction with α-tubulin and localization to mitotic spindle. (A) IB analysis from PBRM1-deficient PBRM1 CRISPR-KO HEK293T cells rescued with GFP-tagged WT or mutant (P1048R, T1202K, or C1233W) PBRM1 constructs showing reduced coimmunoprecipitation of α-tubulin with P1048R and C1233W mutants and respective input lysates. Representative blots (n = 4). (B) IB analysis cells in (A) using GFP-Trap agarose magnetic beads. Representative blots (n = 5). Bottom panels in (A) and (B) shows quantification of the IB analysis for interaction with tubulin (represented as fold change after normalization for tubulin) with means ± SEM. P value was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test for multiple comparisons against WT GFP-PBRM1. (C) Flowchart and graph showing analysis of stacked images of PBRM1-deficient PBRM1 CRISPR-KO HEK293T cells expressing WT or mutated GFP-PBRM1 constructs using MATLAB software. Data are represented as means ± SEM. P values are determined by one-way ANOVA followed by Kruskal-Wallis post hoc test for multiple comparisons against WT GFP-PBRM1. (D) Representative images showing localization of PBRM1 to the mitotic spindle using deconvolution microscopy of PBRM1-deficient PBRM1 CRISPR-KO HEK293T cells ectopically expressing WT or mutant P1048R, T1202K, and C1233W GFP-PBRM1 constructs (green) and stained for α-tubulin (red) and chromosomes (DAPI).
SETD2, access these residues to modify α-tubulin, and how luminal acetyl and methyl marks direct the function of microtubules. Whether SETD2 methylates α-tubulin before or after assembly of α-/β-tubulin dimers into microtubules is unknown. However, ATAT-1 acetylates α-tubulin after assembly into microtubules and is thought to access luminal K40 to acetylate this residue via pores in the microtubule shaft (33, 34). These and other studies have led to the growing appreciation that lattice dynamics of even stable microtubules can provide access to luminal residues along the shaft of microtubule polymers (35–37). Recently, a passive breathing mechanism for microtubules in which α-/β-tubulin dimers can be lost and replaced from the microtubule shaft was proposed (37), which is thought to be facilitated by the severing enzymes katanin and spastin (38). As SWI/SNF complexes are known to evict histones and other proteins from nucleosomes during chromatin remodeling (39), our current study suggests the intriguing hypothesis that PBAF could be playing a similar role in evicting tubulin and/or other microtubule-associated proteins during microtubule remodeling.

The BAH domain crystal structure has been defined for both chicken and human PBRM1 (40), but the function of this domain has not been clearly defined. Our data now confirm a role for PBRM1 BAH domains in recognition of the α-TubK40me3 mark.
Furthermore, while the loss of PBRM1 is known to cause genomic instability (30, 32), we have identified a previously unknown functional role for PBRM1 in the maintenance of genomic stability via binding to microtubules and recruitment of an ATPase-competent PBAF complex to the mitotic spindle. This activity requires the BAH domain(s) of PBRM1 that inhibit binding to methylated α-tubulin to fail to rescue the genomic instability seen with PBRM1 loss despite their ability to form transcriptionally competent PBAF complexes. Recently, the BAH domain of the origin recognition complex (Orc1) in yeast was also shown to be important in maintenance of genomic stability, an activity distinct from its function in transcriptional silencing (41). This BAH domain provides an interface between Orc1 and the nucleosome, and BAH domain mutations that disrupt this interaction increased the frequency of double-strand breaks. Both BAH1 and BAH2 domains of PBRM1 appear to be required for maintenance of genomic stability, as mutations in either BAH1 or BAH2 decreased spindle localization and caused genomic instability. This suggests that there could be cooperativity between these domains for microtubule binding, perhaps due to sequential binding of the two domains to recognize microtubules and recruit PBAF, which contains two PBRM1 molecules for each BRG1 ATPase subunit (3).

Because of their colocalization on chromosome 3p, one allele each of PBRM1 and SETD2 are co-deleted as an early event in development of ccRCC (42), with “second hits” in these epigenetic proteins the second and third most common oncogenic events in this cancer. While PBRM1 mutations occur in excess of 30% of ccRCC, this is far from the only tumor type affected by PBRM1 mutation. Cholangiocarcinoma, for example, has more than 20% mutation rate for this gene. Other tumor types with more than 5% mutation rate include uterine, stomach, melanoma, mesothelioma, and bladder cancer (43, 44). The overall prevalence of PBRM1 is 4.9% (2% as loss of function) across the 11 most frequent tumor types in TCGA (45). These mutations are distributed across the gene, including mutations in the BAH domains (46). Aside from co-occurrence with VHL loss, observed in ccRCC (25), no other known co-occurring mutations have been discovered to date. Notably, mutational exclusivity is observed with other PBAF complex mutations common in cancer (ARID2, BRD7) (45). In a recent evolutionary model of ccRCC developed by the TRACERx study (47, 48), second hits resulting in loss of the reader PBRM1 precede loss of the writer SETD2, but in no case has loss of SETD2 (and its methyl mark) preceded PBRM1 loss. While the reason for this sequence has remained obscure, our results suggest that cancer cells with second hits that inactive SETD2 will lack the cytoskeletal methyl mark read by PBRM1, thereby abrogating PBRM1 cytoskeletal function, and may gain no further (cytoskeletal) advantage by loss of PBRM1. Conversely, while early loss of PBRM1 can contribute to tumor progression by eliminating an α-TubK40me3 reader, later loss of SETD2 will result in loss of both the cytoskeletal α-TubK40me3 and chromatin H3K36me3 marks. Notably, in the setting of loss of PBRM1, repeated subclonal selection for SETD2 mutations occurs (48), suggesting that complete loss of both cytoskeletal and chromatin methyl marks may be an evolutionary bottleneck in cancer progression. Thus, our study identifies dysregulation of microtubule methylation as novel nexus of convergence in explaining how mutations in an epigenetic writer (SETD2) and reader (PBRM1) can drive genomic instability and cancer progression via cytoskeletal defects. This opens new windows into understanding how defects in components of the epigenetic machine with chromatocytoskeletal activities can drive the development of cancer, and perhaps other diseases, via disruption of the cytoskeleton, potentially even independently of disruption of the epigenome.

Last, while epigenetic inheritance is well established, less is known regarding how natural selection acting on environmentally induced phenotypes could drive epimutations and evolution of the epigenome itself (49, 50). In this regard, the concept of an epigenetic machine that coordinates hereditary components of the epigenome with function of the cytoskeleton has interesting implications for evolutionary biology (51). Epigenetic readers, writers, and erasers are responsive to the environment. Examples include the impact of diet on 1-carbon metabolism and availability of methyl donors, regulation of the activity of demethylases by oxygen levels, and kinases that respond to environmental cues, which, when activated, can phosphorylate and modulate the activity of chromatin remodelers (52). Our data suggest that changes in the environment could produce coordinate changes in methylation on both the epigenome and the cytoskeleton, with functional consequences. Evolutionary theory indicates that environmentally induced phenotypes can supplement adaptation by enabling a population to survive in a new or changing environment until potentially adaptive genetic changes become fixed in the population. In the case of evolution of the epigenome, a changing environment could cause coordinated cytoskeletal changes (producing phenotypes upon which natural selection could act) and changes in the epigenome (which could be inherited generationally) (51). This would predict that over evolutionary time, epigenetic marks on chromatin and cytoskeleton could acquire coordinated functions. This is clearly the case for methyl marks made by SETD2, which function on both chromatin and the cytoskeleton to maintain genomic stability [the present report and (14, 15, 32)]. Although still quite speculative, as the dual chromatin...
and cytoskeletal activity of epigenetic readers, writers, and erasers more fully emerges, this hypothesis could provide useful ways to explore evolution of the epigenome and provide insights into how epigenetic variation and phenotypic variation, the actual target of selection, may be linked.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Details of the antibodies used for immunoblotting (IB) and immunofluorescence (IF) are as follows: PBRM1 (Bethyl Laboratories, A301-591A; 1:1000 IB), lactate dehydrogenase (Abcam, ab47010; 1:5000 IB), lamin A/C (Cell Signaling, 2032S; 1:1000 IB), COX5A (Abcam, ab180129; 1:500 IB), DM1A (Santa Cruz Biotechnology, sc32293; 1:4000 IB, IF), GFP (Santa Cruz Biotechnology, sc-9996; 1:5000 IB), p150Glued (BD Biosciences, 610474; 1:400 IB), MAP4 (Abcam, ab89650; 1:800 IB), α-tubulin (Sigma-Aldrich, T6199; 1:4000 IB), α- tubulin (Sigma-Aldrich, T6199; 1:4000 IB), α-tubulin (Thermo Fisher Scientific, PA5-19489; 1:5000 IB), and ARID2 (Santa Cruz Biotechnology, sc-17796; IB), SNF5 (Sigma-Aldrich, SAB420020; 1:1000 IB), ARID2 (Santa Cruz Biotechnology, sc-166117; 1:500 IB), PBRM1 (Boster Bio, M01130; 1:200 IF), BRG1 (Thermo Fisher Scientific, 720129; 1:200 IF), GFP (Santa Cruz Biotechnology, sc-9996; 1:4000 IB), and ARID2 (Novus Biologicals, NBP-2-57220; 1:250 IF) (GFP-Trap magnetic agrose bead, ChromoTek, gtma20). Secondary antibodies (1:5000) conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology. For IF staining, Alexa Fluor–labeled secondary antibodies (1:2000) were purchased from Invitrogen. Nuclear counterstaining reagent, 4′,6-diamidino-2-thidiazene Sepharose beads (DAPI) (1:4000), was purchased from Invitrogen. SlowFade Gold Antifade reagent (Thermo Fisher Scientific, S36937) was used as mounting medium for coverslips.

**Cell culture and transfection**

HEK293T cell lines acquired from Theremo Fisher Scientific (R70007) and HKCs (acquired from W.K.R.’s laboratory, Vanderbilt University, TN) were maintained in Dulbecco’s modified Eagle's medium (DMEM) (Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (FBS). The human 786-O SETD2+/+ and SETD2−/− RCC cell line (acquired from W.K.R., Vanderbilt University, TN) were maintained in DMEM (Life Technologies, 11875-085) supplemented with 10% FBS. MEF cells were maintained in phenol-free DMEM (Gibco, 21063-029) supplemented with 10% FBS, sodium pyruvate (Gibco, 11360070), and GlutaMAX (Gibco, 35050061). FBS used to supplement the medium was purchased from Sigma-Aldrich (F24429). The transient transfection of plasmids and siRNAs was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific, 11668-500) and Dharmafect-1 (Dharmacon, T-2001-03), respectively, according to the manufacturer’s instructions. All of the cell lines used in this study were routinely tested and confirmed negative for mycoplasma.

**Cloning, site-directed mutagenesis, and plasmid DNA preparation**

GFP-BRG1 (Addgene, 65391) and GFP-PBRM1 (Addgene, 65387) plasmids were gifts from K. Miller (53). GFP-PBRM1 construct served as a template to make single-point mutants of GFP-PBRM1 (P1048R, T1202K, and C1233W) using the QuikChange II XL Site-Directed Mutagenesis Kit and confirm these mutations with Sanger sequencing. All the six bromodomains and two BAH domains were cloned in pGEX-4T-1 vector (Millipore Sigma, GE28-9545-4) for GST pull-down assays using In-Fusion HD cloning. Bromodomains and BAH domains corresponded to amino acids 31 to 169 (BD1), 162 to 348 (BD2), 341 to 512 (BD3), 497 to 661 (BD4), 654 to 784 (BD5), 776 to 903 (BD6), 950 to 1080 (BAH1), and 1150 to 1280 (BAH2). All the primers for cloning were designed using SnapGene software.

**GST-tagged protein purification**

For purification of GST-tagged PBRM1 domain proteins, BL21 DE3 cells were transformed with 50 ng of DNA of each of the domain constructs, plated, and incubated at 37°C overnight. Single colonies were picked to inoculate 5-ml LB (primary inoculum) medium and allowed to grow at 37°C overnight. Two milliliters of overnight primary culture was then inoculated into 200 ml of LB medium and allowed to grow at 37°C until the OD (optical density) reached to 0.6 to 0.8. Once the OD was in between 0.6 and 0.8, the culture was induced by adding isopropyl-β-d-thiogalactopyranoside to achieve a final concentration of 0.5 mM. This culture was allowed to grow at 25°C with 160 rpm speed for another 5 hours. Cells were harvested after 5 hours of induction, and the pellet was stored at −80°C. The pellet was resuspended in 10 volume of resuspension buffer [50 mM tris (pH 8.0), 150 mM NaCl, 0.1% Triton X-100, phenylmethylsulfonyl fluoride, and protease inhibitor cocktail Roche] followed by bacterial lysis with microfluidizer LM20. The lysed solution was cleared by centrifugation at 15,000g for 20 min. Glutathione Sepharose beads were added to the cleared solution and incubated on a rocker for 4 hours to allow the beads to bind to protein. After the incubation, beads were washed with resuspension buffer with salt gradient. Last, the beads were resuspended in phosphate-buffered saline (PBS) with protease inhibitor and used for experiments.

**GST pull-down assays**

Expression and purity of purified GST bromodomain and BAH domain protein attached to the glutathione Sepharose beads were analyzed by SDS–polycrylamide gel electrophoresis (PAGE) gel stained with Coomassie blue. For GST pull-down assay, the glutathione Sepharose beads with bromodomain or BAH domain were washed with Cell Signaling Technology (CST) buffer [20 mM tris (pH, 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1× cOmplete protease inhibitor cocktail Roche] and incubated with 1 µg of porcine brain tubulin (Cytoskeleton, T238P) for 4 hours at room temperature. After 4-hour incubation, the beads were washed three times 10 min each using high-salt buffer (50 mM tris, 300 mM NaCl, 1 mM EDTA, and 1% NP-40). The sample was heated in a 1× denaturing loading buffer for 10 min at 95°C before being loaded into SDS-PAGE (4 to 15%). IB analyses were performed using a mouse anti–α-tubulin antibody.

**siRNA knockdown of PBRM1**

ON-TARGETplus SMARTpool Human-PBRM1 siRNA (L-008692-01-5) and ON-TARGETplus Non-Targeting pool (D-001810-10-20) were purchased from Dharmacon. siRNAs were resuspended in 1× siRNA buffer (GE Dharmacon) to obtain 20 µM stock. HEK293T cells were...
transfected with indicated siRNA at 10 nM final concentration with Dharmafect-1 according to the manufacturer’s instructions for 72 hours before lysis collection or before fixing cells for immunostaining.

Generation of PBRM1-KO HEK293T stable cell lines using CRISPR-Cas9 and validation

PBRM1-KO HEK293T cells were generated using guide RNA with Cas9 construct purchased from GenScript. Guide RNA sequence (GAAACCACCTTCATAATAGTC) was designed on exon 4. Cells were transfected with Cas9 construct for 48 hours followed by puromycin selection (2 mg/ml) for 1 week to isolate nontarget (NT) and/or PBRM1-KO HEK293T cells. Single colonies were picked using clonal rings after 1 week of selection and screened using a genomic DNA preparation, and primers were upstream and downstream from the cut site, followed by polymerase chain reaction and sequencing. Clones were further confirmed for loss of PBRM1 using Western blotting.

Whole-cell lysis and subcellular fractionation

To make whole-cell lysates, cells (70 to 80% confluent) were washed and collected by scraping into ice-cold PBS, pelleted by centrifugation at 4°C, resuspended in 1× lysis buffer [20 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1× cOmplete protease inhibitor cocktail (Roche)], sonicated for five cycles with 30 s on and 30 s off per cycle, and centrifuged at maximum speed for 10 min at 4°C. The pellet was removed, and supernatants were collected as whole-cell extracts for IB and immunoprecipitation analysis for soluble proteins. For subcellular fractionation, the pellet, collected after centrifugation of scraped cells as mentioned above, was resuspended in a hypotonic buffer [10 mM Hepes (pH 7.2), 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 20 mM NaF, and 100 μM Na2VO4] and disrupted by hand homogenizer for 15 to 20 times. Disruption of the cells was confirmed using a hemocytometer. Following homogenization, samples were centrifuged at 10,000 rpm with supernatant collected as “cytoplasmic fraction” and pellets were layered on prewarmed microtubule cushion (5% sucrose in 1× PBS) and centrifuged at 120,000 g for 1 hour. The supernatant was collected and mixed with 5× SDS-PAGE sample buffer. Microtubule pellet was resuspended in 1× SDS-PAGE sample buffer. Lysate containing approximately 36 μg of proteins was used as input, and the corresponding amount of supernatant and pellet fractions was resolved on an 8% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and stained with Ponceau S before Western blotting analysis. The primary antibodies used included anti-PBRM1 rabbit polyclonal antibody, anti-p150Glue mouse monoclonal antibody, anti-MAP4 rabbit polyclonal antibody, and anti-COX5A rabbit monoclonal antibody.

Peptide pull-down assay

The K40 α-tubulin peptides were custom-synthesized from Thermo Fisher Scientific (K40: IQPDGQMPSDKTIGGGDDSFT; K40ac: IQPDGQMPSD [Kac] TIGGGDDSFT; K40me3: IQPDGQMPSD [Kme3] TIGGGDDSFT) with biotin label at the C terminus. The peptide pull-down assays were performed as described previously by Porter and Dykhuizen (20), with some modifications. The 20-μl streptavidin agarose resins (Thermo Fisher Scientific) were washed three times with binding buffer [0.5 mM dithiothreitol (DTT), 150 mM NaCl, 50 mM tris] and resuspended in 300 μl of binding buffer with 2 μg of biotin-labeled tubulin peptide (K40 only, K40-Ac, and K40-me3) (Thermo Fisher Scientific), and samples were rotated at 4°C for 2 hours. Cells were harvested and lysed in immunoprecipitation buffer [25 mM tris (pH 8), 300 mM NaCl, 1% NP-40, 1 mM EDTA, plus protease inhibitors] and sonicated for five cycles using 30 s on and 30 s off. The samples were then spun down at 10,000 g for 10 min. The lysate (300 μl) was added to the peptide and resin solution and rotated overnight. The samples were washed for 10 min three times in IP buffer. The resin was resuspended in 1× sample buffer and boiled for 7 min at 95°C. The samples were run on 4 to 15% SDS-PAGE gel (Bio-Rad) and processed for IB analysis.

Immuno precipitation and IB analysis

For immunoprecipitation, whole-cell lysates or fractionated cytoplasmic lysates were incubated with indicated antibodies and magnetic A/G beads (Thermo Fisher Scientific) or GFP-Trap overnight at 4°C. The beads were pelleted and washed with cell lysis buffer three times and were heated in 1× denaturing loading buffer for 10 min at 95°C before being loaded into SDS-PAGE. The cell lysates were separated on a 4 to 15% gel (Bio-Rad), transferred to polyvinylidine difluoride membranes, and probed with respective antibodies. Densitometric analysis for quantification of expression levels was performed using ImageQuant TL software, and data were normalized with α-tubulin.

Microtubule co-sedimentation assay

Microtubule co-sedimentation assay was performed as described previously by Miller et al. (54), with some modifications. HEK293T wild-type and PBRM1 CRISPR-KO cells were harvested and rinsed in PBS twice. Cells were resuspended in BRB80 [80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂ (pH 6.8)] supplemented with protease inhibitor cocktail (cOmplete Mini EDTA-free, Roche) and 1 mM DTT. After sonication for 40 s, the lysate was centrifuged at 120,000 g at 4°C for an hour. To assemble microtubules, the cleared lysate was incubated at 37°C for 25 min in the presence of 10 mM taxol and 1 mM guanosine 5′-triphosphate (GTP). Microtubule-containing lysates were layered on prewarmed microtubule cushion (5% sucrose in BRB80 supplemented with 10 mM taxol and 1 mM GTP) and centrifuged at 80,000 g at 37°C for 30 min. Supernatant was collected and mixed with 5× SDS-PAGE sample buffer. Microtubule pellet was resuspended in 1× SDS-PAGE sample buffer. Lysate containing approximately 36 μg of proteins was used as input, and the corresponding amount of supernatant and pellet fractions was resolved on an 8% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and stained with Ponceau S before Western blotting analysis. The primary antibodies used included anti-PBRM1 rabbit polyclonal antibody, anti-p150Glue mouse monoclonal antibody, anti-MAP4 rabbit polyclonal antibody, and anti-COX5A rabbit monoclonal antibody.

IF staining and imaging

Cells seeded on coverslips were fixed either by immersion in cold methanol at −20°C for 5 min followed by rehydration in PBS for 5 min or with 4% paraformaldehyde (PFA) in PBS at room temperature for 15 min followed by permeabilization with 0.5% Triton X-100 in PBS (PBST) for 20 min. Fixed cells were then blocked with 3.75% bovine serum albumin (BSA) in PBS for 1 hour and incubated in primary antibodies diluted in 3.75% BSA overnight at 4°C. Primary
antibodies used for IF analysis included rabbit anti-PBRM1, rabbit anti-BRG1 antibody, mouse anti-α-tubulin antibody, rabbit anti-α-tubulin, and rabbit anti-ARID2 antibody. Following three washes of 10 min each using 1× PBS, cells were incubated with corresponding Alexa Fluor–labeled secondary antibodies (Invitrogen, Eugene, OR) at a dilution of 1:2000 at room temperature for an hour. Cells were then washed three times with 1× PBS, with 10-min incubation in each wash, and then postfixied for 10 min using 4% PFA to stabilize the signal. Cells were then counterstained with DAPI (Invitrogen; diluted 1:4000) for 10 min to visualize the DNA. Coverslips were mounted in SlowFade Gold Antifade Mountant (Invitrogen, S36937). Fixed cells were imaged with a CFI Plan Apochromat Lambda 60× oil, 1.4 numerical aperture (NA) objective, and DS-Qi2 camera and mounted on Nikon Eclipse Ti2-E inverted research microscope (Nikon Instruments, Melville, NY) equipped for standard phase contrast and epifluorescence microscopy, as well as for deconvolution. Image acquisition was carried out using an Andor Zyla 4.2+ sCMOS high-sensitivity monochrome camera and was driven by Nikon NIS-Elements Advanced Research (AR) image acquisition and analysis software. Acquired images were exported for advanced three-dimensional (3D) and 2D deconvolution modules for improved image quality. Eight-bit images were exported, and figures were prepared using Photoshop version CC software (Adobe Systems, Mountain View, CA). To determine the colocalization and intensity of signal, a line was drawn between two spindle pole using deconvolution microscope NIS elements software. Graphical representations and statistical analyses were performed using GraphPad Prism software version 8. Cell phenotypes were scored visually by counting nonoverlapping fields in a raster pattern across the coverslip using the Eclipse Nikon Ti2-E inverted research microscope.

For live-cell imaging, cells were plated on 35-mm glass-bottomed microwell petri dish (MatTek Corporation, Ashland, MA) at a confluence of 5 × 10^5 and co-reverse–transfected with 2 μg of GFP-PBRM1 and pBT097-pAAV EfHa-H2BmRuby2 using Lipofectamine 2000 (Life Technologies) for 48 hours in a 37°C incubator supplied with 5% CO2. Then, before imaging, the medium in the dish was changed to phenol-free complete DMEM supplemented with 10% FBS to ensure no fluorescence interference from phenol. The culture dish was then placed into temperature-controlled stage (Tokai PBRM1 and pBT097-pAAV EfHa-H2BmRuby2 using Lipofectamine 2000 (Life Technologies) for 48 hours in a 37°C incubator supplied with 5% CO2. Then, before imaging, the medium in the dish was changed to phenol-free complete DMEM supplemented with 10% FBS to ensure no fluorescence interference from phenol. The culture dish was then placed into temperature-controlled stage (Tokai Hit, Shizuoka-ken, Japan) prewarmed to 37°C and supplied with 5% CO2. Z-stacks were acquired every 1 to 2 min using a CFI Plan Apochromat Lambda 100× oil, NA = 1.45, mounted on a Nikon Eclipse Ti2-E with Yokogawa W1 Spinning Disk Confocal Microscope and Photometrics Prime 95B sCMOS camera with NIS-Elements AR software. EDF (extended depth of focus) projections of the images were then generated. Measurement of fluorescence intensity was carried out using Nikon’s NIS Element software.

**Image analysis using MATLAB software**

**Image registration and overlays**

After cropping of microscopy images to preserve the whole-cell bodies of mitotic cells, three-color (DAPI, GFP, Texas Red) fluorescence deconvolution images were aligned using a two-step image registration procedure described below: Because spindle poles of individual cells were readily visible, radial alignment began by selecting the two-pixel locations corresponding to the spindle poles. Images were then rotated such that the spindle pole axis was horizontal, with the resulting rotated images contained in a new image size of 1024 pixels wide by 1024 pixels tall. With respect to cell size, all images were scaled in size so that the distance between spindle poles corresponded to 256 pixels, with all spindle poles in the same positions in all images. The resulting aligned images permitted image stacking based on summing normalized pixel intensity values at each position.

**Fluorescence color normalization**

To ensure that all images counted equally in summed aligned images, each channel was normalized by dividing pixel intensities (arbitrary units) over the median (background) pixel intensity. The resulting signal-to-background ratios were used for downstream analysis.

**Intensity profiles and intensity calculations**

Fluorescence intensity profiles for all channels were obtained by integrating the signal-to-background ratios using a 10-pixel tall region across the line connecting the two spindle poles. To assess the fluorescence intensity at the spindle poles, the fluorescence signal-to-background intensity values were summed in a 10-pixel wide by 10-pixel high box centered at the locations of the spindle poles.

**RNA sequencing**

For transcriptional profiling, library preparation and sequencing was performed as previously described (55). Briefly, HEK293T CRISPR-KO PBRM1 cells were collected after 48-hour transfection of either GFP-PBRM1 or mutant P1048R, T1202K, and C1233W and grown for 48 hours. Total RNA was extracted using TRIzol reagent (Ambion, Life Technologies) and purified using RNasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. RNA samples underwent quality control assessment using the RNA tape on TapeStation 4200 (Agilent) and were quantified with Qubit Fluorimeter (Thermo Fisher Scientific). RNA libraries were prepared and sequenced at the University of Houston Seq-N-Edit Core per standard protocols. RNA libraries were prepared with the QIAseq Stranded Total RNA Library Kit (Qiagen) using 100 ng of input RNA. Ribosomal RNA depletion was performed with the QIAseq Fast Select rRNA Removal Kit (Qiagen). The size selection for libraries was performed using SPRINT select beads (Beckman Coulter), and purity of the libraries was analyzed using the High Sensitivity D1000 tape on TapeStation 4200 (Agilent). The prepared libraries were pooled and sequenced using NextSeq 500 (Illumina), generating ~15 million 2 × 76–base pair paired-end reads per sample.

Transcriptomic analysis was performed as previously described (55). Paired-end reads were trimmed using TrimGalore and mapped to the UCSC hg38 genome build using HISAT2. Aligned reads were counted against the Gencode gene model annotation (56) to obtain expression values by using FeatureCounts (57). Differential gene expression was evaluated using the R package edgeR (58), with TMM (trimmed means of m values) normalization. Significance was achieved for a fold change exceeding 1.5× and an adjusted P value of <0.05. We sequenced 15 million to 16 million read pairs per sample after trimming of low-quality base pairs.

**Over-representation analysis**

Over-representation analysis was performed to detect enrichment of gene sets corresponding to pathways and biological processes, based on differential gene expression. Using the Hallmark compendium (v6.2) and the Molecular Signature Database methodology (MSigDB), a hypergeometric test was used to assess the enrichment, with significance achieved at P < 0.05.
Mass spectrometric analysis
After immunoprecipitation, the washed beads were boiled in 1× NuPAGE LDS sample buffer (Invitrogen), subjected to NuPAGE 10% bis-tris gel (Invitrogen), visualized with Coomassie Brilliant blue staining, and excised into gel pieces according to molecular size. The excised SDS-PAGE gel piece was subject to in-gel digestion using trypsin (GenDepot, T9600) after destaining. The in-gel digested peptides were resuspended in loading solution (5% methanol containing 0.1% formic acid) and injected to a nano-LC 1000 system (Thermo Fisher Scientific) coupled to a Q Exactive Plus (Thermo Fisher Scientific) mass spectrometer. An in-house pre-system (Thermo Fisher Scientific) coupled to a Q Exactive Plus coupled to a nano-LC 1000 system (Thermo Fisher Scientific) coupled to a Q Exactive Plus and injected to a nano-LC 1000 system (Thermo Fisher Scientific) coupled to a Q Exactive Plus and used to enrich and separate the peptides. The peptides were eluted using 60 min of discontinuous gradient of 4 to 26% acetonitrile/0.1% formic acid at a flow rate of 700 nl/min. The mass spectrometer was operated in the data-dependent acquisition mode acquiring fragmentation spectra of the top 25 strongest ions and under the direct control of Xcalibur software (Thermo Fisher Scientific). Obtained MS/MS spectra were searched against target-decoy human refseq database in Proteome Discoverer 2.1 interface (Thermo Fisher Scientific) with Mascot mascot algorithm (Mascot 2.4, Matrix Science). The precursor mass tolerance was confined within 20 ppm, with fragment mass tolerance of 0.02 dalton and a maximum of two missed cleavage allowed. Dynamic modification of oxidation, protein N-terminal acetylation, and destreak were allowed. The peptides identified from the mascot result file were validated with a 5% false discovery rate (FDR). Gene quantification was performed using the label-free, intensity-based absolute quantification (iBAQ) approach by “gpGrouper” algorithm as previously described (59, 60). Two-tailed Student’s t test using two-sample equal variance was used to determine the statistically significant protein.

Structural analysis of BAH1 and BAH2 domains
The available crystal structure of human BAH1 (PDB: 6OXB) was used as a reference model to gain insight into possible structural changes because of amino acid mutations P1048R in BAH1 and C1233W and T1202K in BAH2 that have been reported in the TCGA database for ccRCC and biochemically characterized in our studies. The amino acid substitutions, corresponding to the mutations, in the structure were made using PyMOL software (pymol.org) and structurally analyzed. To understand the structural consequences of mutations in BAH2, we generated a structural model of BAH2 using Swiss modeler server that automatically chose the human BAH1 structure as a template. A structure-based sequence alignment of BAH1 and BAH2 sequences was further carried out using T-Coffee software to confirm the choice of BAH1 structure as a reference model.

Statistical analysis
All the data values are represented as the SEM. GraphPad Prism 7.0 software was used for data analysis. Statistical significance was determined by either Student’s t test or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Unless otherwise noted, every experiment was done with at least three biologically independent replicates. P value of less than 0.05 was considered statistically significant. Representative Western blots and microscopy images were shown from at least three biologically independent replicates that showed similar results.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/14/eabf2866/DC1

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