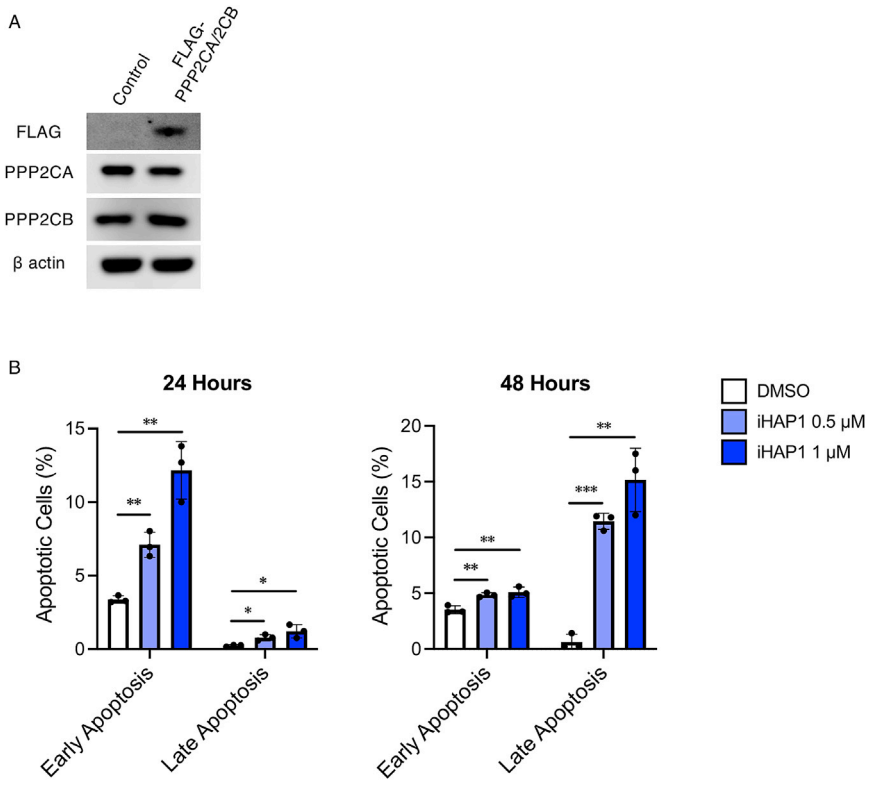

Figure S1. Bioactivity of iHAP1, a Selective and Potent Activator of PP2A, Related to Figure 1

(A) The protein expression levels of FLAG-tagged PPP2CA (N terminus) and FLAG-tagged PPP2CB (N terminus) were detected in the lysates from KOTP-K1 cells that stably express these constructs. Cell lysates were immunoblotted with indicated antibodies.

(B) The percentages of apoptotic cells reflecting early (Annexin V+ and PI-) and late apoptotic (Annexin V+ and PI+) cell death after iHAP1 treatment for 24 and 48 hours. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Student's t test, comparing the means ± SD of three biological replicates versus controls.

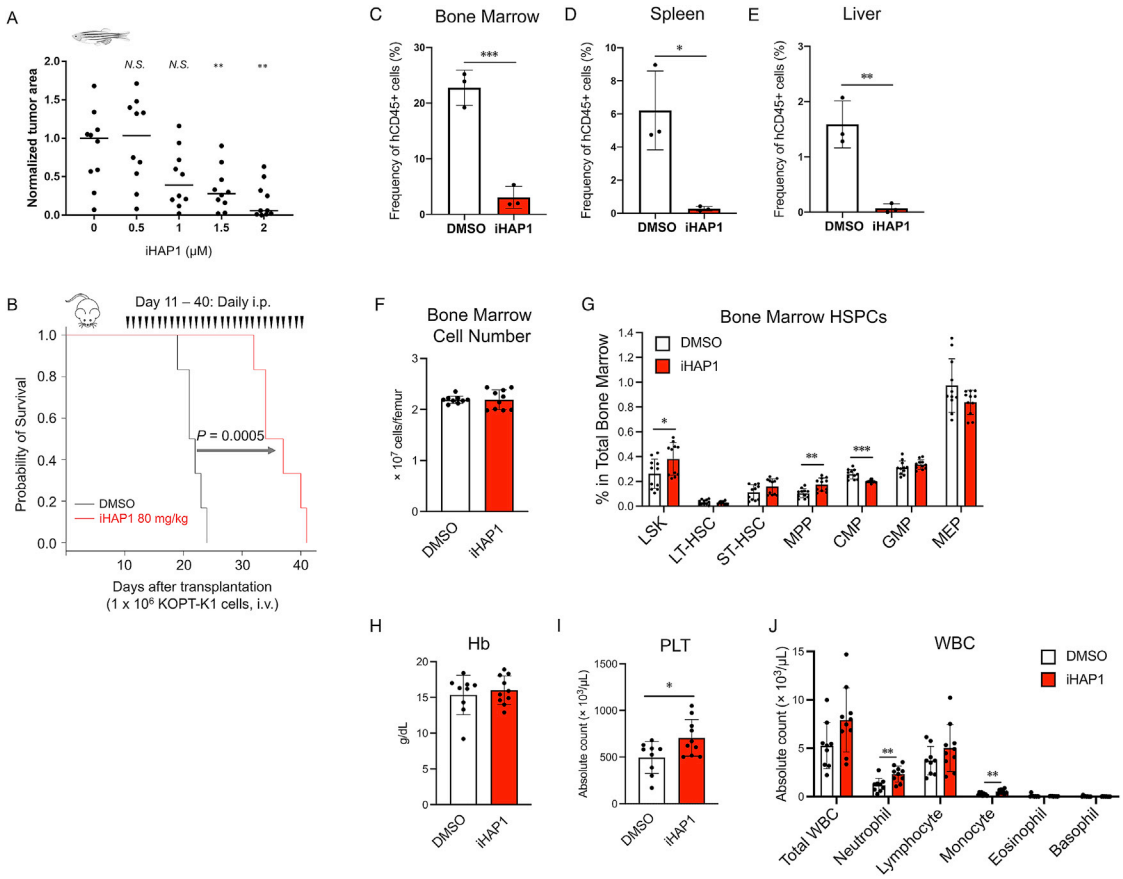

Figure S2. Antitumor Activity *In Vivo* of iHAP1 versus PPZ in T-ALL Preclinical Models, Related to Figure 2

(A) Quantification of GFP-positive leukemic area in zebrafish embryos treated with increasing doses of iHAP1, compared with DMSO control (n = 10 for each dose). **p < 0.01 and ****p < 0.0001 by two-tailed Welch's t test, black bars indicate median values. *N.S.*, not significant.

(B) Kaplan–Meier survival analysis of NSG mice xenotransplanted with 1 million KOPT-K1 human T-ALL cells (day 1). Treatment with iHAP1 80 mg/kg/day or vehicle control (daily i.p. injections; 5% DMSO in 10% 2-hydroxypropyl-β-cyclodextrin) began on day 11. Survival times were recorded when the mice were sacrificed with signs of leukemia development (ruffled fur, swelling or lack of activity). DMSO versus iHAP1 (80 mg/kg), p = 0.0005. Six mice were tested in each cohort; p values were determined with the log-rank test.

(C–E) Flow cytometric assessment of the percentages of CD45+ human T-ALL cells in (C) bone marrow, (D) spleen and (E) liver of iHAP1-treated T-ALL xenografts at 18 days post-xenotransplant, and 24 hours after mice received the last of seven daily doses of iHAP1 (80 mg/kg/day, i.p.) compared with vehicle controls. *p < 0.05, **p < 0.01 and ***p < 0.001 by Student's t test, comparing the means ± SD of three biological replicates versus controls.

(F and G) iHAP1 treatment minimally affects the hematopoietic stem and progenitor cells in mice. C57BL/6 mice were treated with either iHAP1 (80 mg/kg/day i.p.) or DMSO control every 24 hours for seven days, and sacrificed twenty-four hours after the last treatment. (F) The numbers of bone marrow cells recovered by flushing per femur (n = 10 for each cohort) were unchanged by iHAP1 treatment. (G) The percentages of long-term hematopoietic stem cells (LT-HSC), short-term HSC (ST-HSC), multipotent progenitors (MPP), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) were examined in the bone marrow using flow cytometry for iHAP1 treated compared to vehicle control mice (n = 11 for each cohort). *p < 0.05, **p < 0.01 and ***p < 0.001 by Student's t test, comparing the means ± SD.

(H–J) The complete peripheral blood counts from C57BL/6 mice treated with iHAP1 compared to vehicle control (n = 9 for vehicle and n = 10 for iHAP1). (H) Hb; hemoglobin levels, (I) PLT; platelet counts, and (J) WBC; white blood cell counts. *p < 0.05 and **p < 0.01 by Student's t test, comparing the means ± SD.

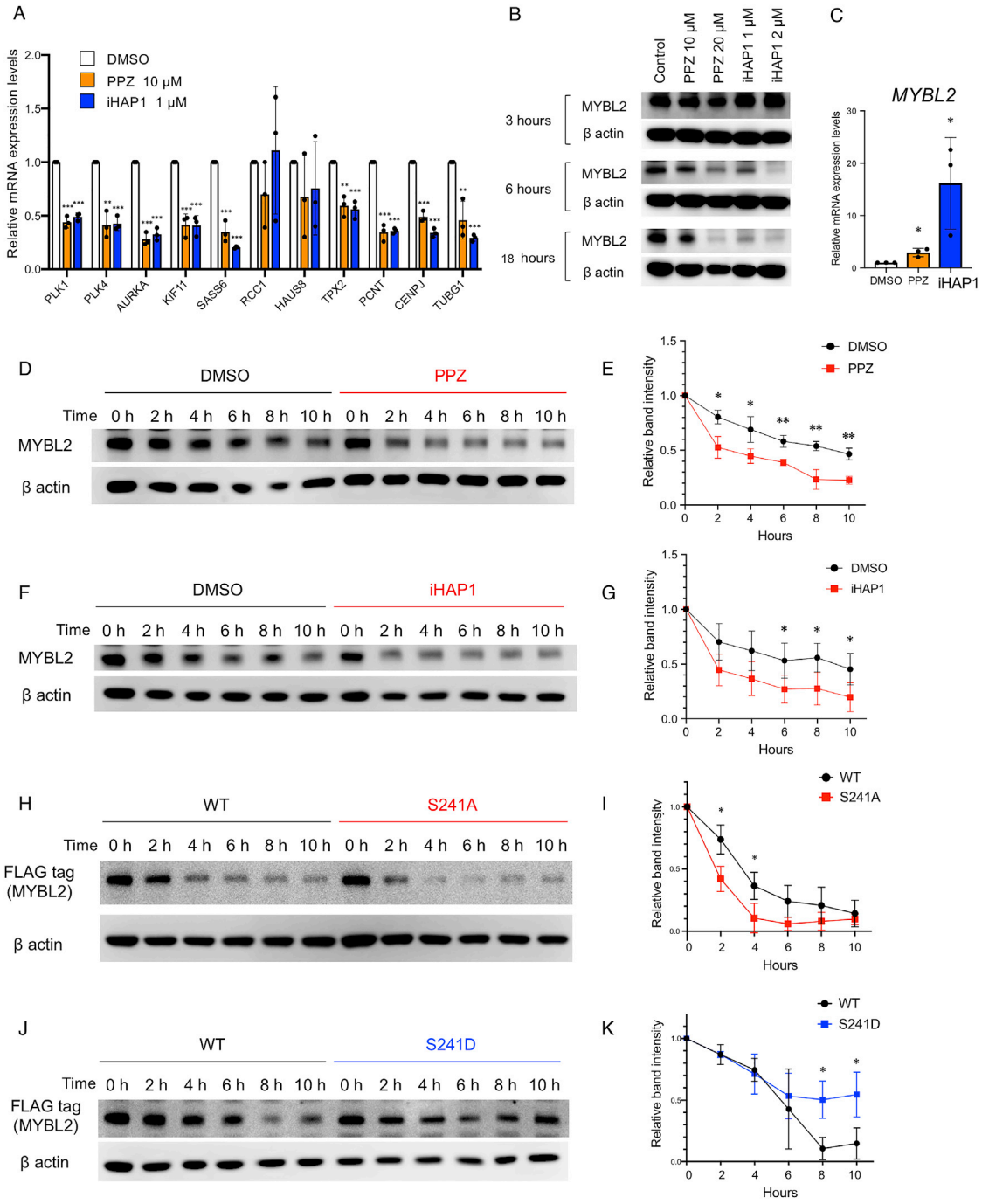

Figure S3. Dephosphorylation of p-MYBL2 S241 by the PP2A Complex that Is Activated by PPZ/iHAP1 Treatment Leads to MYBL2 Degradation in KOPT-K1 Cells, Related to Figure 3

(A) The relative mRNA expression levels of genes whose inducible CRISPR-cas9 knockout causes cell cycle arrest in prometaphase with spindle monopolarity (*PLK1, PLK4, AURKA, KIF11, SASS6, RCC1, HAUS8, TPX2, PCNT, CENPJ* and *TUBG1* (McKinley and Cheeseman, 2017) were evaluated in KOPT-K1 cells treated with DMSO control, PPZ (10 μM) or iHAP1 (1 μM) for 6 hours. PPZ and iHAP1 treatment significantly downregulated the expression levels of most of these genes. **p < 0.01 and ***p < 0.001 versus controls by Student's t test; the data are means ± SD of three biological replicates.

(B) western blots showing the expression levels of MYBL2 in KOPT-K1 cells at serial times during PPZ/iHAP1 treatments. Cells were treated with either PPZ (10 or 20 μM) or iHAP1 (1 or 2 μM) for the indicated times before protein extraction. Expression levels of the indicated proteins were analyzed by western blotting.

(C) The relative mRNA expression levels of MYBL2 were evaluated in KOPT-K1 cells treated either by DMSO, PPZ (10 μM) or iHAP1 (1 μM) for three hours.

(D) Time-course assays with cycloheximide (CHX) to block new protein synthesis to determine the half-life of the MYBL2 protein. KOPT-K1 cells were simultaneously treated with 10 μM PPZ and 100 μg/ml CHX for the indicated times before protein extraction. MYBL2 was detected by western blotting.

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(E) Relative band intensity levels of MYBL2 in (D) are shown, normalized to the corresponding levels before treatment. *$p < 0.05$, **$p < 0.01$ versus controls by Student's t test; the data are means ± SD of three biological replicates.
(F) Time-course assays in the presence of CHX to determine the half-life of the MYBL2 protein. KOPT-K1 cells were simultaneously treated with 1 μM iHAP1 and 100 μg/ml CHX for the indicated times before protein extraction. MYBL2 was detected by western blotting.
(G) Relative band intensity levels of MYBL2 in (F) are shown, normalized to the corresponding levels before treatment. *$p < 0.05$ versus controls by Student's t test; the data are means ± SD of three biological replicates.
(H) Time-course assays in the presence of CHX to determine the half-life of the FLAG-tagged wild-type (WT) and S241A mutant MYBL2 proteins. KOPT-K1 cells expressing either FLAG-tagged WT or S241A mutant MYBL2 proteins were treated with 100 μg/ml CHX for the indicated times before protein extraction. MYBL2 was detected by western blot using anti-FLAG tag antibody.
(I) Relative band intensity levels of MYBL2 in (H) are shown normalized to the corresponding levels before treatment. *$p < 0.05$ versus controls by Student's t test; the data are means ± SD of three biological replicates.
(J) Time-course assays in the presence of CHX to determine the half-life of the FLAG-tagged wild-type (WT) and S241D mutant MYBL2 proteins. KOPT-K1 cells expressing either FLAG-tagged WT or S241D mutant MYBL2 proteins were treated with 100 μg/ml CHX for the indicated times before protein extraction. MYBL2 was detected by western blot using anti-FLAG tag antibody.
(K) Relative band intensity levels of MYBL2 in (J) are shown, normalized to the corresponding levels before treatment. *$p < 0.05$ versus controls by Student's t test; the data are means ± SD of three biological replicates.

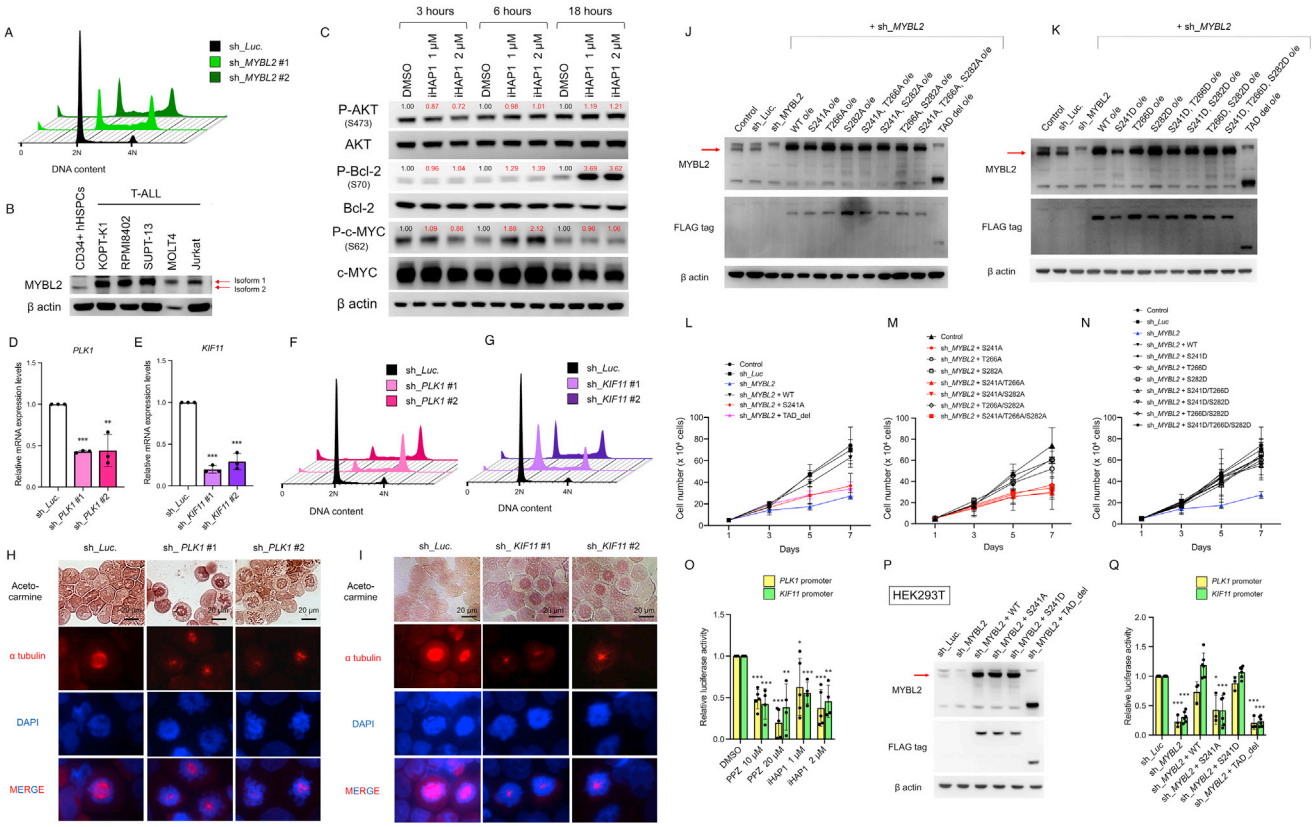

Figure S4. Identification of Ser241 of MYBL2 as a Critical Phosphorylation Site Mediating Its Transactivation Activity, Related to Figure 4

(A) After *MYBL2* knockdown in KOPT-K1 cells, the relative DNA content of cells in each of the samples was determined by flow cytometric analysis of propidium iodide staining. shRNAs targeting control *luciferase* (sh_*Luc*.) or *MYBL2* (sh_*MYBL2* #1 and #2) were induced with 3 μM doxycycline for 48 hours. Knockdown of MYBL2 induced substantial G2/M phase arrest in the cell cycle.

(B) western blots showing the expression levels of MYBL2 in CD34+ human hematopoietic stem and progenitor cells (HSPCs) and five different T-ALL cell lines (KOPT-K1, RPMI8402, SUPT-13, MOLT4 and Jurkat cells). The two isoforms of human MYBL2 expressed in these cell lines have been reported by other investigators, who showed that the slower migrating isoform 1 is the full length transactivating form of this transcription factor, while the isoform 2 represents a splice variant lacking transactivating domain and functioning to interfere with target gene activation (Horstmann et al., 2000).

(C) Dephosphorylation of potential substrates of PP2A during iHAP1 treatment was examined in KOPT-K1 cells. Cells were treated with iHAP1 (1 or 2 μM) for 3 hours, 6 hours and 18 hours, then phosphorylation status of AKT, Bcl-2 and c-MYC was measured by immunoblotting. p-AKT S473 was not affected by iHAP1 treatment at any time point, while p-Bcl-2 S70 was unaffected until 18 hours, when it was dramatically increased. p-c-MYC S62 was mainly unaffected, although it did increase slightly after treatment for 6 hours. For MYBL2, we have shown that p-MYBL2 S241 is markedly dephosphorylated after 3 hours of treatment as shown by phosphoproteomics analysis in response to both iHAP1 and PPZ (Figures 4A and 4B). Although we do not yet have an antibody specific for p-MYBL2 S241, we have shown that overall levels of the protein drop precipitously at 6 and 18 hours in a dose-dependent manner after treatment with either drug (Figure S3B). These results indicate that the antitumor activity of iHAP1 is primarily mediated by dephosphorylation of MYBL2, but not by other known substrates of PP2A, such as c-MYC, AKT or Bcl-2.

(D and E) The relative mRNA expressions of *PLK1* and *KIF11* were evaluated in KOPT-K1 cells expressing shRNAs that targeted control *luciferase* (sh_*Luc*.) or (D) *PLK1* (sh_*PLK1* #1 and #2) and (E) *KIF11* (sh_*KIF11* #1 and #2). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus controls by Student's t test; the data are means ± SD of three biological replicates.

(F and G) Two different target genes of MYBL2 were knocked down in KOPT-K1 cells and the relative DNA content of cells in each of the samples was determined by flow cytometric analysis of propidium iodide staining. shRNAs targeting control *luciferase* (sh_*Luc*.), (F) *PLK1* (sh_*PLK1* #1 and #2) and (G) *KIF11* (sh_*KIF11* #1 and #2) were induced with 3 μM doxycycline for 48 hours. Knockdown of *PLK1* or *KIF11* induced substantial G2/M phase arrest in the cell cycle, as indicated with increased cells with 4N DNA content.

(H and I) Morphologic assessment was conducted in KOPT-K1 cells expressing shRNAs targeting control *luciferase* (sh_*Luc*.) or (H) *PLK1* (sh_*PLK1* #1 and #2) and (I) *KIF11* (sh_*KIF11* #1 and #2). Acetocarmine, AlexaFluor 647 (red) anti-α-tubulin antibody and DAPI were used to stain chromatin, microtubules and DNA, respectively. In sharp contrast to the control KOPT-K1 cells, knockdown of *PLK1* and *KIF11* with 3 μM doxycycline for 48 hours induced prometaphase arrest in the cell cycle, producing cells in which the spindle and associated microtubules exhibited monopolarity. We also observed a minority of prometaphase cells exhibiting spindle multipolarity in cells after knockdown of *PLK1* expression (McKinley and Cheeseman, 2017).

(J) Levels of protein expression of MYBL2 after shRNA-mediated knockdown and the nonphosphorylatable alanine mutants of MYBL2 (S241A, T266A, S282A, S241A/T266A, S241A/S282A, T266A/S282A and S241A/T266A/S282A), together with the transcriptional activation domain-deleted MYBL2 mutant (TAD_del), were detected in the lysates from KOTP-K1 cells. Expression of shRNAs and mutant MYBL2 constructs was induced by 3 μM doxycycline for 48 hours. Note that the mutant MYBL2 constructs harbor FLAG tags to discriminate them from endogenous MYBL2. Cell lysates were immunoblotted with the indicated antibodies.

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(K) The protein expression of MYBL2 after shRNA-mediated knockdown and the phosphomimetic aspartic acid mutants of MYBL2 (S241D, T266D, S282D, S241D/T266D, S241D/S282D, T266D/S282D and S241D/T266D/S282D), together with transcriptional activation domain-deleted MYBL2 mutant (TAD_del), was detected in the lysates from KOTP-K1 cells. Expressions of shRNAs and mutant MYBL2 constructs were induced by 3 μM doxycycline for 48 hours. Note that the mutant MYBL2 constructs harbor FLAG tags to discriminate them from the endogenous MYBL2. Cell lysates were immunoblotted with the indicated antibodies.
(L) Cell proliferation curves of KOPT-K1 cells with or without shRNA-mediated *MYBL2* knockdown. *MYBL2* gene knockdown led to a significant reduction in cell growth rate, suggesting a major role for MYBL2 in supporting cell proliferation. To test whether phosphorylation of MYBL2 Ser241 is required for cell growth, we conducted rescue experiments after *MYBL2* shRNA-mediated knockdown. WT MYBL2 successfully rescued the shMYBL2-induced suppression of cell growth, while neither a nonphosphorylatable alanine mutant of MYBL2 at Ser241 (S241A) nor transcriptional activation domain-deleted MYBL2 mutant (TAD_del) could rescue this phenotype. Thus the transcriptional activation domain of MYBL2, and phosphorylation of MYBL2 S241 in this domain, are critically important for the function of this gene.
(M) Identification of phospho-S241 as a critical site mediating the growth inhibition effects of MYBL2. In rescue experiments of the cell growth effects of shRNA-induced downregulation of *MYBL2,* we tested a series of nonphosphorylatable alanine mutants of MYBL2 (S241A, T266A, S282A, S241A/T266A, S241A/S282A, T266A/S282A and S241A/T266A/S282A). Mutant MYBL2 harboring T266A, S282A or T266A/S282A reverted the sh_MYBL2-induced suppression of cell growth, indicating that phosphorylation of these serines or threonines in the transcriptional activation domain of MYBL2 was not required for its ability to support cell cycle progression and cell growth. By contrast, mutant MYBL2 harboring S241A, S241A/T266A, S241A/S282A or S241A/T266A/S282A mutants could not rescue the cell growth phenotype, indicating that phospho-S241 in the transcriptional activation domain of MYBL2 was the only phosphorylated site in the TAD that was important to rescue the growth arrest of KOPT-K1 cells induced by knockdown of *MYBL2*.
(N) Cell growth rescue attempts with phosphomimetic aspartic acid mutants of MYBL2 (S241D, T266D, S282D, S241D/T266D, S241D/S282D, T266D/S282D and S241D/T266D/S282D). Restoring MYBL2 expression using these mutants successfully reverted the sh_MYBL2-induced suppression of cell growth, indicating that phosphorylation of these serines in the transcriptional activation domain of MYBL2 did not alter the ability of the factor to support cell cycle progression and cell growth.
(O) The relative activities of promoters were determined for two representative MYBL2 target genes, *PLK1* and *KIF11*, each of which when knocked down caused cell cycle arrest in prometaphase yielding spindle monopolarity (McKinley and Cheeseman, 2017). HEK293T cells were transiently transfected with a vector expressing *luciferase* under control of either the *PLK1* promoter or the *KIF11* promoter. The activities of the promoters were measured by detecting luminescence. HEK293T cells were treated with PPZ or iHAP1 at the indicated concentrations for three hours. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus DMSO control by Student's t test; the data are means ± SD of three biologic replicates.
(P) Protein expression levels were detected in cell lysates from transfected HEK293T cells after shRNA-mediated knockdown of endogenous MYBL2 and FLAG-tagged rescue constructs including WT MYBL2, a nonphosphorylatable alanine mutant of MYBL2 at Ser241 (S241A), a phosphomimetic aspartic acid mutant of MYBL2 at Ser241 (S241D) and a transcriptional activation domain-deleted mutant of MYBL2 (TAD_del). Expression of shRNAs and WT or mutant *MYBL2* constructs were induced by 3 μM doxycycline for 48 hours. Cell lysates were immunoblotted with the indicated antibodies.
(Q) The relative activities of promoters were determined for two representative MYBL2 target genes, *PLK1* and *KIF11*, each of which when knocked down caused cell cycle arrest in prometaphase yielding spindle monopolarity (McKinley and Cheeseman, 2017). HEK293T cells were transiently transfected with a vector expressing *luciferase* under control of either the *PLK1* promoter or the *KIF11* promoter. The activities of the promoters were measured by detecting luminescence. Endogenous *MYBL2* was knocked down using gene-specific shRNAs targeting the 3′ UTR, and then its expression was restored by simultaneous overexpression of WT *MYBL2* or a series of mutant *MYBL2* constructs (S241A, S241D or TAD_del). Expression of both shRNAs and MYBL2 constructs were induced by 3 μM doxycycline for 24 hours. *MYBL2* knockdown led to downregulation of the activity of these promoters, which was restored to control levels by WT *MYBL2* or mutant *MYBL2* S241D, but not by mutant *MYBL2* S241A or TAD_del, underscoring the importance of MYBL2 S241 phosphorylation for the function of MYBL2 as a transcription factor. *$p < 0.05$ and ***$p < 0.001$ versus sh_Luc. control by Student's t test. The data are means ± SD of three biologic replicates.

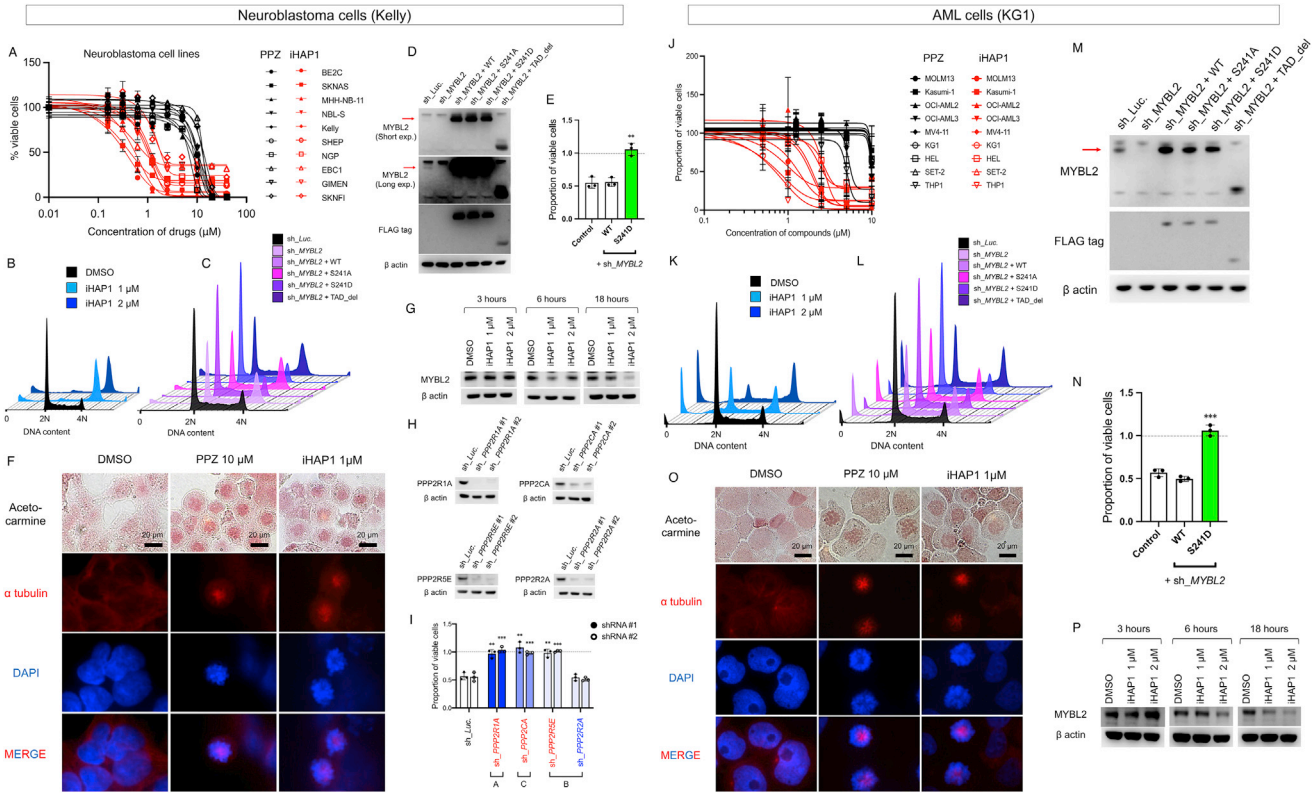

Figure S5. Phosphorylation of Ser241 on MYBL2 Is Necessary and Sufficient for Antitumor Activity of iHAP1 in Neuroblastoma-Derived Kelly and AML-Derived KG1 Cells, Related to Figure 5

(A) Dose-response curves of PPZ and iHAP1 in 10 different human neuroblastoma cell lines; BE2C, SKNAS, MHH-NB-11, NBL-S, Kelly, SHEP, NGP, EBC1, GIMEN and SKNFI. Cells were treated with PPZ or iHAP1 at various concentrations for 72 hours, before cell viability was examined with PrestoBlue® reagent. Data are presented as the means ± SD of three biologic replicates.

(B) Kelly cells were treated with DMSO (control) or iHAP1 for 24 hours. The relative DNA content of cells in each of the samples was determined by flow cytometric analysis of propidium iodide staining. Treatment with iHAP1 (1 μM and 2 μM) induced G2/M phase arrest in the cell cycle, as indicated by an increased number of cells with 4N DNA content.

(C) Cell cycle effects of *MYBL2* shRNA knockdown with simultaneous overexpression of WT *MYBL2*, mutant *MYBL2* S241A (nonphosphorylatable alanine mutant), S241D (a phosphomimetic aspartic acid mutant) or a transcriptional activation domain-deleted mutant of MYBL2 (TAD_del) were determined in Kelly cells. Expression of both the shRNAs and *MYBL2* constructs was induced with 3 μM doxycycline for 48 hours, and relative cellular DNA content in each of the samples was measured by flow cytometric analysis of propidium iodide staining. DNA histogram shows the cell cycle status of Kelly cells after inducible *MYBL2* knockdown with *MYBL2*-specific shRNA, demonstrating arrest of the cells in G2/M phase of the cell cycle with 4N DNA content. This G2/M phase cell cycle arrest was rescued by both WT MYBL2 and the phosphomimetic mutant MYBL2 S241D, but not by the nonphosphorylatable mutant MYBL2 S241A or the transcriptional-activation-domain deletion (*MYBL2* TAD_del).

(D) The protein expression levels of MYBL2 were detected in the lysates from Kelly cells after shRNA-mediated knockdown and simultaneous overexpression of WT MYBL2, MYBL2 S241A, MYBL2 S241D and MYBL2 TAD_del. Expression of shRNAs and the WT or mutant MYBL2 constructs was induced by 3 μM doxycycline for 48 hours. The overexpressed *MYBL2* constructs harbor a FLAG tag to distinguish them from the endogenous *MYBL2* protein. Cell lysates were immunoblotted with the indicated antibodies.

(E) Sensitivity to iHAP1 was tested in Kelly cells that expressed the phosphomimetic aspartic acid mutant form of MYBL2. In MYBL2 knock-down cells, either the wild-type (WT) or mutant MYBL2 gene (S241D) was expressed. Cells were treated with 0.5 μM iHAP1 for 72 hours, then examined for viability. **$p < 0.01$ by Student's t test, comparing the means ± SD of 3 biological replicates versus controls.

(F) Kelly cells were treated with DMSO (control), PPZ (10 μM) or iHAP1 (1 μM) for 24 hours. Acetocarmine, AlexaFluor 647 (red) anti-α tubulin antibody and DAPI were used to stain chromatin, microtubules and DNA, respectively. In sharp contrast to the control cells, PPZ and iHAP1 treatment induced prometaphase arrest of the cell cycle, resulting in cells that exhibited monopolarity of the centrioles and an associated starburst of microtubules.

(G) western blots showing the expression levels of MYBL2 in Kelly cells upon iHAP1 treatment. Cells were treated with iHAP1 at 1 or 2 μM for the indicated hours before protein extraction.

(H) western blots showing the expression levels of each of the subunits of PP2A in Kelly cells conditionally expressing shRNAs specifically targeting PPP2R1A, PPP2CA, PPP2R5E (B56ε) or PPP2R2A (B55α). Expression of shRNA constructs was induced by 3 μM doxycycline for 48 hours before protein extraction.

(I) Kelly cells with selective PP2A subunit downregulation were tested for their sensitivity to iHAP1. Each PP2A subunit was knocked down by shRNAs unique for each subunit. Control shRNA targeted the *luciferase* gene. Cells were pretreated with 3 μM doxycycline for 48 hours for shRNA expression, then treated with 0.5 μM iHAP1 for 72 hours before examining cell viability. **$p < 0.01$ and ***$p < 0.001$ versus control by Student's t test; the data are means ± SD of three biological replicates.

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(J) Dose-response curves of PPZ and iHAP1 in nine different human AML cell lines: MOLM13, Kasumi-1, OCI-AML2, OCI-AML3, MV4-11, KG1, HEL, SET-2 and THP1. Cells were treated with PPZ or iHAP1 at various concentrations for 72 hours, before their viability was determined with PrestoBlue® reagent. Data are the means ± SD of three biologic replicates.

(K) KG1 cells were treated with DMSO (control) or iHAP1 for 24 hours. The relative DNA content of cells in each of the samples was determined by flow cytometric analysis of propidium iodide staining. Treatment with iHAP1 (1 μM and 2 μM) induced G2/M phase arrest in the cell cycle, as indicated by an increased number of cells with 4N DNA content.

(L) Cell cycle effects of *MYBL2* shRNA knockdown were determined in KG1 cells with simultaneous overexpression of WT *MYBL2*, or mutants *MYBL2* S241A, S241D or TAD_del. Expression of both the shRNAs and *MYBL2* constructs were induced by 3 μM doxycycline for 48 hours, and relative cellular DNA content in each of the samples was measured by flow cytometric analysis of propidium iodide staining. DNA histogram shows the cell cycle status of KG1 cells after inducible *MYBL2* knockdown with gene-specific shRNA, demonstrating arrest of the cells in G2/M phase of the cell cycle with 4N DNA content. This G2/M phase cell cycle arrest was rescued by both WT MYBL2 and the phosphomimetic mutant MYBL2 S241D, but not by MYBL2 S241A or *MYBL2* TAD_del.

(M) The protein expression levels of MYBL2 were detected in the lysates of KG1 cells after shRNA-mediated knockdown and simultaneous overexpression of WT MYBL2, MYBL2 S241A, MYBL2 S241D and MYBL2 TAD_del. shRNA expression was induced by adding 3 μM doxycycline for 48 hours. The overexpressed *MYBL2* constructs harbor FLAG tags to distinguish them from the endogenous *MYBL2* protein. Cell lysates were immunoblotted with the indicated antibodies.

(N) Sensitivity to iHAP1 was tested in KG1 cells with the phosphomimetic aspartic acid mutant forms of MYBL2. In MYBL2 knocked down cells, each of the wild-type (WT) or mutant MYBL2 gene (S241D) were additionally expressed. The control shRNA targeted luciferase gene (*sh_Luc*). Cells were treated with 0.5 μM iHAP1 for 72 hours, then examined for viability. ***$p < 0.001$ by Student's t test, comparing the means ± SD of 3 biological replicates versus controls.

(O) KG1 cells were treated with DMSO (control), PPZ (10 μM) or iHAP1 (1 μM) for 24 hours. Acetocarmine, AlexaFluor 647 (red) anti-α tubulin antibody and DAPI were used to stain chromatin, microtubules and DNA, respectively. In sharp contrast to the control cells, PPZ and iHAP1 treatment induced prometaphase arrest of the cell cycle, producing cells in which the spindle and associated microtubules exhibited monopolarity.

(P) western blots showing the expression levels of MYBL2 in KG1 cells at serial times during iHAP1 treatment. Cells were treated with iHAP1 at 1 or 2 μM for the indicated times before protein extraction.

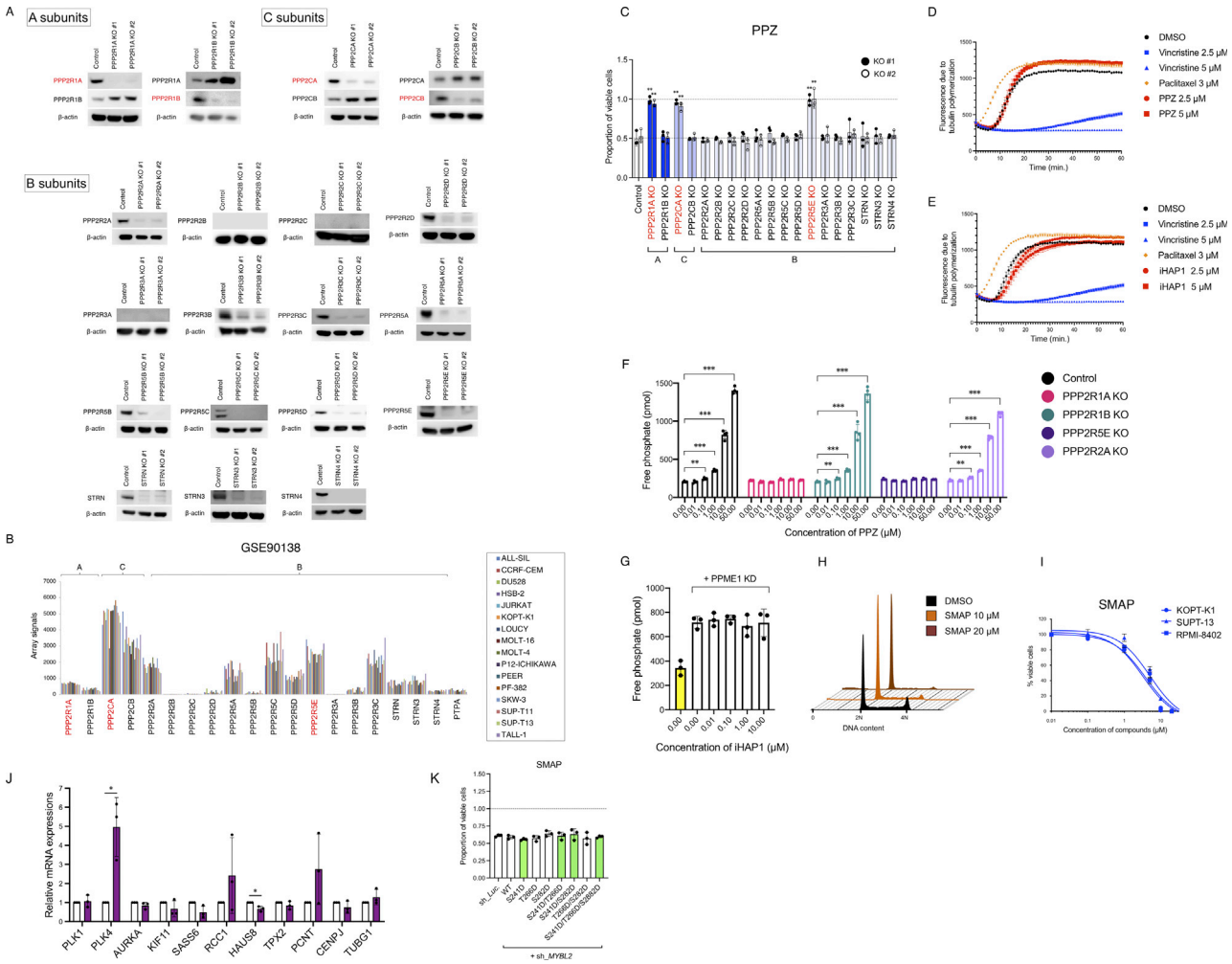

Figure S6. Identification of the Essential Subunits of PP2A Required for Phosphatase Activation and Antitumor Activities of iHAP1 and SMAP, Related to Figure 6

(A) western blots showing that each of the PP2A A, B and C subunits was knocked out by CRISPR-Cas9 in KOPT-K1 cells with two independent gRNAs with different target sequences for each PP2A subunit (see Table S2 for the targeting sequences). Control gRNAs target the luciferase gene. The basal expression levels of PPP2R2B (B55β), PPP2R2C (B55γ) and PPP2R3A (PR72) were undetectable, consistent with the very low mRNA expression levels of these subunit genes in T-ALL cell lines (see Figure S6B). Note that the anti-PPP2R5C Monoclonal Antibody (TQ11-1G6) detects isoforms gamma-3 and gamma-2, but not the C terminus lacking isoform gamma-1. Both gRNAs target sequences common to all three isoforms of PPP2R5C (B56γ).

(B) mRNA expression levels of individual PP2A subunits in 16 different T-ALL cell lines. Relative expression levels correspond to the array signal intensities of probes identifying the subunits (GSE90138).

(C) Sensitivity to PPZ was tested in KOPT-K1 cells with selective PP2A subunit inactivation. Each subunit was knocked out by CRISPR-Cas9 using two unique gRNAs designed for each subunit (#1 and #2; Figure S6A). Control gRNA targeted the luciferase gene. Cells were treated with PPZ at 5 μM for 72 hours then examined for viability. **p < 0.01 by Student's t test, comparing the means ± SD of three biological replicates versus controls.

(D and E) Results obtained from a fluorescence-based tubulin polymerization assay performed with (D) PPZ and (E) iHAP1 at the indicated concentrations. Paclitaxel at 3 μM and vincristine at 2.5 and 5 μM were simultaneously tested as controls. Paclitaxel stabilizes microtubules and thus accentuates tubulin polymerization, while vincristine inhibits tubulin polymerization into microtubules. PPZ and iHAP1 did not interfere with tubulin polymerization in this assay. This experiment was conducted with a tubulin polymerization assay kit, produced by Cytoskeleton, Inc., that employs porcine tubulin protein that is > 99% pure to assess tubulin polymerization. PPZ and iHAP1 do not block tubulin polymerization or otherwise affect microtubule assembly up to 2.5 or 5 μM, respectively, while vincristine markedly inhibited tubulin polymerization at 3 μM.

(F) Phosphatase activity of PP2A in control KOPT-K1 cells versus KOPT-K1 cells with selective PP2A subunit inactivation. Cells were treated with PPZ at various concentrations for 3 hours. KO indicates knockout. Values shown as means ± SD of four biological replicates versus controls. **p < 0.01 and ***p < 0.001 by Student's t test.

(G) For reference, the first bar (yellow) shows the phosphatase activity using the universal substrate of PP2A immunoprecipitated with anti-FLAG tag antibody from KOPT-K1 cells expressing FLAG-tagged PPP2CA and PPP2CB. IP-western blotting shows that these PP2A enzymes contain the STRN, STRN3 and STRN4 subunits (Figure 6C). To drive formation of the PP2A-B56ε enzyme in KOPT-K1 cells we knocked down PPME1 using a doxycline inducible *PPME1* specific shRNA, which allows LCMT1 to methylate the C subunit on Lys309 (See Figures 7 and S7). After PPME1 knockdown for 24 hours, PP2A-B56ε was immunoprecipitated with anti-PPP2R5E antibody, and the immunoprecipitates were treated with iHAP1 at graded concentrations for one hour. Control DMSO treated

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preformed PP2A-B56ε enzyme exhibited substantial phosphatase activity when tested with the univeral substrate under these conditions (second bar). Increasing concentrations of iHAP1 treatment did not affect the phosphatase activity of preformed PP2A-B56ε complexes (Bars 3 to 6). Thus, iHAP1 acts exclusively to drive assembly of trimeric PP2A-B56ε in cells expressing C subunits that lack Lys309 methylation and does not have measurable effects on the activity of preformed PP2A-B56ε complexes. The data are means ± SD of three replicates.

(H) KOPT-K1 cells were treated with SMAP or DMSO control for 24 hours. Relative DNA content of cells in each of the samples was determined by flow cytometric analysis of propidium iodide staining. Treatment with SMAP (10 μM and 20 μM) induced G0/G1 phase arrest in the cell cycle, as indicated by the increased number of cells with 2N DNA content.

(I) Dose-response curves for SMAP after 72 hours of treatment in three different T-ALL cell lines - KOPT-K1, RPMI-8402 and SUPT-13 cells. Data are reported as the means ± SD of three biological replicates.

(J) The relative mRNA expression levels of genes whose inducible CRISPR-cas9 knockout causes cell cycle arrest in prometaphase yielding spindle monopolarity (PLK1, PLK4, AURKA, KIF11, SASS6, RCC1, HAUS8, TPX2, PCNT, CENPJ and TUBG1 (McKinley and Cheeseman, 2017) were evaluated in KOPT-K1 cells treated with DMSO control or SMAP (10 μM) for 6 hours. SMAP treatment downregulated HAUS8 expression levels by about 25%, but otherwise did not significantly reduce the expression levels of these genes. *$p < 0.05$ versus controls by Student's t test; the data are means ± SD of three biological replicates.

(K) Sensitivity to SMAP was tested in KOPT-K1 cells with the phosphomimetic aspartic acid mutant forms of MYBL2. In MYBL2 knocked down cells, each of the WT or mutant *MYBL2* genes (S241D, T266D, S282D, S241D/T266D, S241D/S282D, T266D/S282D and S241D/T266D/S282D) were additionally expressed. shRNA-targeted *luciferase* (*sh_Luc*) served as the control. Cells were treated with 5 μM SMAP for 72 hours and then examined for viability.

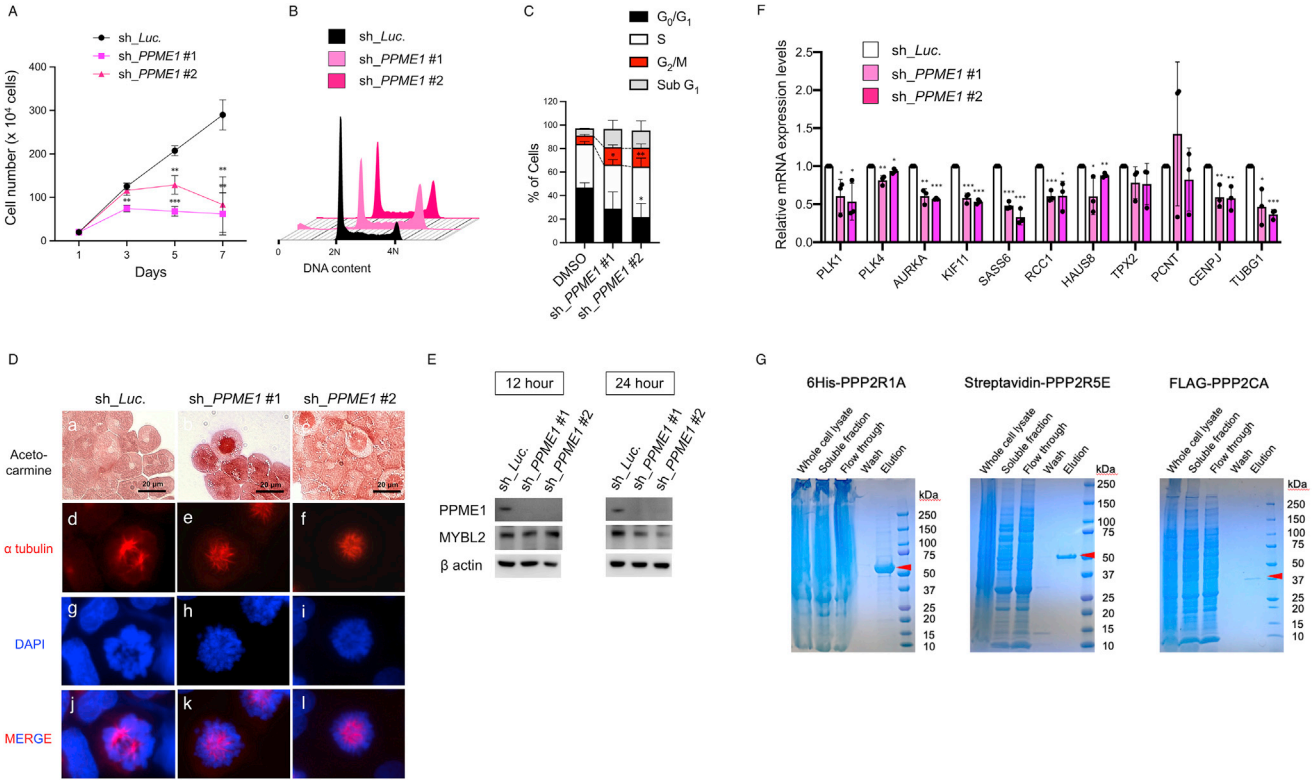

Figure S7. Inhibition of PPME1, an Endogenous Inhibitory Protein for PP2A, Induces Prometaphase Cell Cycle Arrest in T-ALL Cells through Formation of the PP2A Holoenzyme Containing B56 Family Proteins as Regulatory B Subunits, Related to Figure 7

(A) Cell proliferation curves were plotted for KOPT-K1 cells expressing shRNAs targeting control luciferase (sh_*Luc*.) or *PPME1* (sh_*PPME1* #1 and #2). Knockdown of PPME1 by inducing *PPME1* shRNAs with 3 μM doxycycline significantly suppressed the growth of these cells. **p < 0.01 and ***p < 0.001 by Student's t test, comparing the means ± SD of three biological replicates versus controls.

(B) PPME1 was knocked down in KOPT-K1 cells and the relative DNA content of cells in each of the samples was determined by measuring PI (propidium iodide) staining using flow cytometry. shRNAs targeting *PPME1* (sh_*PPME1* #1 and #2) or luciferase control (sh_*Luc*.) were induced with 3 μM doxycycline for 48 hours. Knockdown of PPME1 induced significant G2/M phase arrest in the cell cycle, as indicated by an increased percentage of cells with 4N DNA content compared to control cells.

(C) Means ± SD of 3 biological replicates of the percentage of cells in each phase of the cell cycle for the DNA histograms shown in (B). *p < 0.05 and **p < 0.01 by Student's t test.

(D) Morphologic assessment was conducted in KOPT-K1 cells expressing shRNAs targeting control luciferase (sh_*Luc*.) or *PPME1* (sh_*PPME1* #1 and #2). For immunofluorescence staining, Alexa Fluor 647 (red)-anti-α tubulin antibody and DAPI were used to stain microtubules and DNA, respectively. In sharp contrast to the control KOPT-K1 cells (a, d, g and i), knockdown of PPME1 induced with 3 μM doxycycline for 48 hours caused prometaphase arrest of the cell cycle (b, e, h and k for sh_*PPME* #1, and c, f, i and l for sh_*PPME* #2), producing cells in which the spindle and associated microtubules exhibited monopolarity.

(E) western blots showing the expression levels of MYBL2 in KOPT-K1 cells expressing shRNAs targeting control luciferase (sh_*Luc*.) or *PPME1* (sh_*PPME1* #1 and #2). Expression of shRNAs was induced by 3 μM doxycycline treatment for the indicated hours before protein extraction.

(F) The relative mRNA expression of genes whose inducible CRISPR-cas9 knockout causes cell cycle arrest in prometaphase yielding spindle monopolarity (PLK1, PLK4, AURKA, KIF11, SASS6, RCC1, HAUS8, TPX2, PCNT, CENPJ and TUBG1) (McKinley and Cheeseman, 2017) was evaluated in KOPT-K1 cells after PPME1 knockdown. KOPT-K1 cells expressing sh_*Luc*. or sh_*PPME1* #1 or #2 were treated with 3 μM doxycycline for 24 hours. Knockdown of PPME1 significantly downregulated the expression levels of most of these genes. *p < 0.05, **p < 0.01 and ***p < 0.001 by Student's t test, comparing the means ± SD of three biological replicates versus controls.

(G) The purity of 6His-tagged PPP2R1A (N terminus), Streptavidin-tagged PPP2R5E (B56ε) (N terminus) and FLAG-tagged PPP2CA (N terminus) proteins prepared for the experiment shown in Figures 7D and 7E was examined by Coomassie staining. The cDNA constructs of tagged subunits were subcloned into pAC8 baculovirus expression vectors for insect cell expression. pAC8-6His-PPP2R1A, pAC8-Streptavidin- PPP2R5E (B56ε) or pAC8-FLAG-PPP2CA were co-transfected with linearized baculovirus DNA into Sf9 insect cells for baculovirus production. Collected virus was used to infect High Five insect cells for protein expression, and the expressed subunit proteins were isolated by immunoprecipitation. The purity of the products was verified using the Coomassie stained gels.