SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1

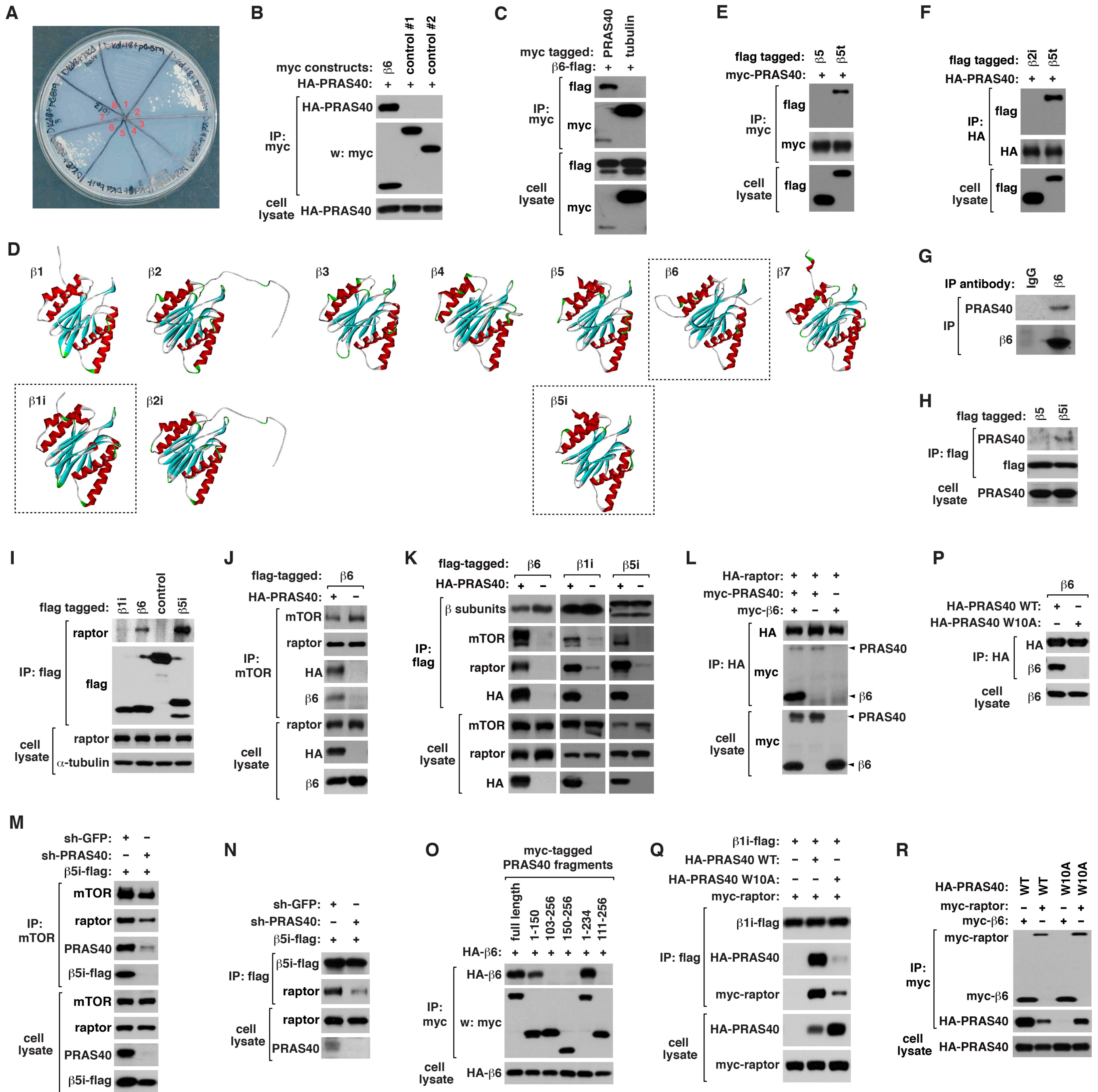

Figure S1, related to Figure 1. mTORC1 binds to β6, β1i, β5i and β5t.

(**A**) Yeast two-hybrid assay of the interaction between β6 and PRAS40. A high stringent condition confirms clone #2 that encodes β6, while excluding cone #6 that shows self-interaction.

(**B**) Confirmation of the interaction between β6 and PRAS40. HA-PRAS40 was coexpressed with myc-tagged β6, S6K1 (control#1), or tubulin (control #2) in HEK293T cells. Myc immunoprecipitates (IPs) were analyzed by western blotting (WB).

(**C**) Confirmation of the interaction between recombinant β6 and PRAS40 in HEK293T cells.

(**D**) Proteasome β subunits show the structural homology. The β subunit structures are from the mouse c-proteasome (PDB code: 3UNE) and the mouse i-proteasome (PDB code: 3UNH). The β subunits capable of binding to mTORC1 are in boxes.

(**E**) β5t, but not β5, interacts with PRAS40. Flag-tagged β5 or β5t was coexpressed with myc-PRAS40 in HEK293T cells.

(**F**) β5t, but not β2i, interacts with PRAS40. Flag-tagged β2i or β5t was coexpressed with myc-PRAS40 in HEK293T cells.

(**G**) Endogenous PRAS40 interacts with endogenous β6. Anti-β6 IPs were prepared from HEK293T cells, and the amount of endogenous PRAS40 was analyzed by WB.

(**H**) Endogenous PRAS40 interacts with β5i but not with β5. Flag-tagged β5 or β5i was transiently expressed in HCT116 cells. Endogenous PRAS40 isolated with flag IPs was analyzed by WB.

(**I**) Endogenous raptor interacts with β1i, β5i and β6. Flag-tagged β1i, β5i, β6 and S6K1 (control) were transiently expressed in HEK293T cells. Endogenous raptor isolated with flag IPs was analyzed by WB.

(**J**) Endogenous mTOR interacts with β6 via PRAS40. Flag-tagged β6 was transiently expressed with or without HA-PRAS40 in HEK293T cells. The amount of β6 recovered with mTOR IPs was analyzed by WB.

(**K**) PRAS40 is important for the interaction between mTORC1 and the i-proteasomal β subunits. Flag-tagged β subunits were expressed with or without HA-PRAS40 in HEK293T cells. Endogenous mTOR and raptor recovered with flag IPs were analyzed by WB.

(**L**) The interaction between raptor and β6 requires PRAS40. The indicated proteins were transiently expressed in HEK293T cells. The amounts of myc-β6 and myc-PRAS40 recovered with HA-raptor were analyzed by WB.

(**M**) PRAS40 knockdown reduces the interaction between mTOR and β5i. Flag-β5i was transiently expressed in HCT116 cells that had been stably transduced by PRAS40 shRNA (sh-PRAS40) or a control shRNA (sh-GFP).

(**N**) PRAS40 knockdown reduces the interaction between raptor and β5i. Flag-β5i was expressed in HCT116 cells that had been stably transduced by PRAS40 shRNA or GFP shRNA. The amount of raptor recovered with β5i-flag was analyzed by WB.

(**O**) An N-terminal region of PRAS40 is important for binding to β6. Myc-tagged PRAS40 fragments were expressed with HA-tagged β6 in HEK293T cells.

(**P**) PRAS40 W10A mutation disrupts the interaction between PRAS40 and β6. Myc-β6 was expressed with HA-tagged PRAS40 WT or W10A in HEK293T cells. The amount of β6 isolated with HA IPs was analyzed by WB.

(**Q**) PRAS40 W10A mutation disrupts the interaction between β1i and raptor. The indicated proteins were transiently expressed in HEK293T cells.

(**R**) PRAS40 W10A mutation disrupts the interaction between PRAS40 and β6, but not the interaction between PRAS40 and raptor. Myc-tagged β6 and raptor were transiently expressed with HA-tagged WT or W10A PRAS40 in HEK293T cells.

Figure S2, related to Figure 2

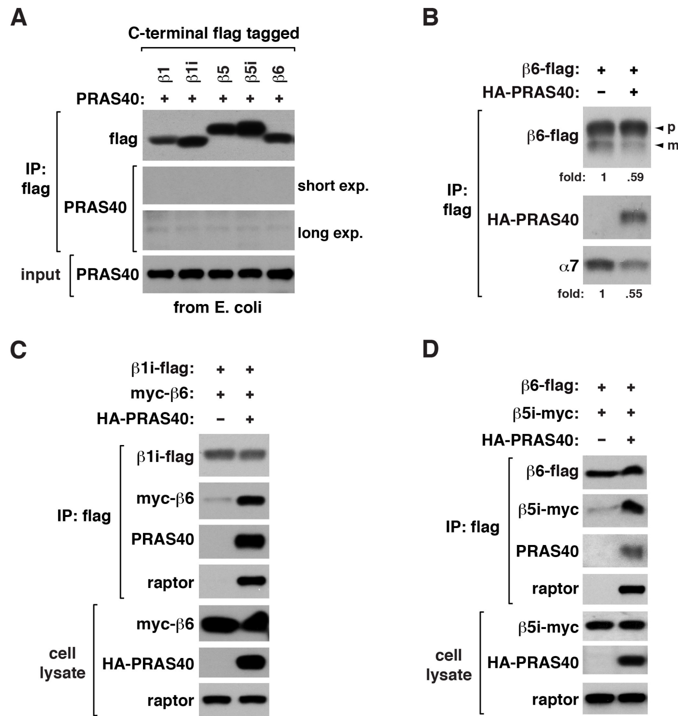

Figure S2, related to Figure 2. PRAS40 binds to the precursors of β1i, β5i and β6 during their folding and suppresses de novo assembly of the 20S core.

(**A**) β1i, β5i and β6 precursors did not bind to PRAS40 in vitro when they were separately expressed. Flag-tagged β subunit precursors were expressed in E. coli, and cell extract was incubated with PRAS40 that was purified from E. coli. Flag IPs were analyzed for the interaction.

(**B**) PRAS40 suppresses de novo biogenesis of proteasomes. The indicated proteins were transiently expressed in HEK293T cells. The amount of α7 recovered associated with the recombinant β6-flag was analyzed by immunoprecipitation and WB. "p" and "m" indicate the precursor and mature forms, respectively.

(**C**) PRAS40 enhances the β1i-β6 interaction. The indicated proteins were transiently expressed in HEK293T cells.

(**D**) PRAS40 enhances the β6-β5i interaction. The experiment was conducted as in (C) except that β6-flag and β5i-myc were expressed instead. The same data is also presented in Figure 7D when WT PRAS40 is compared with mutant PRAS40 constructs.

Figure S3, related to Figure 3

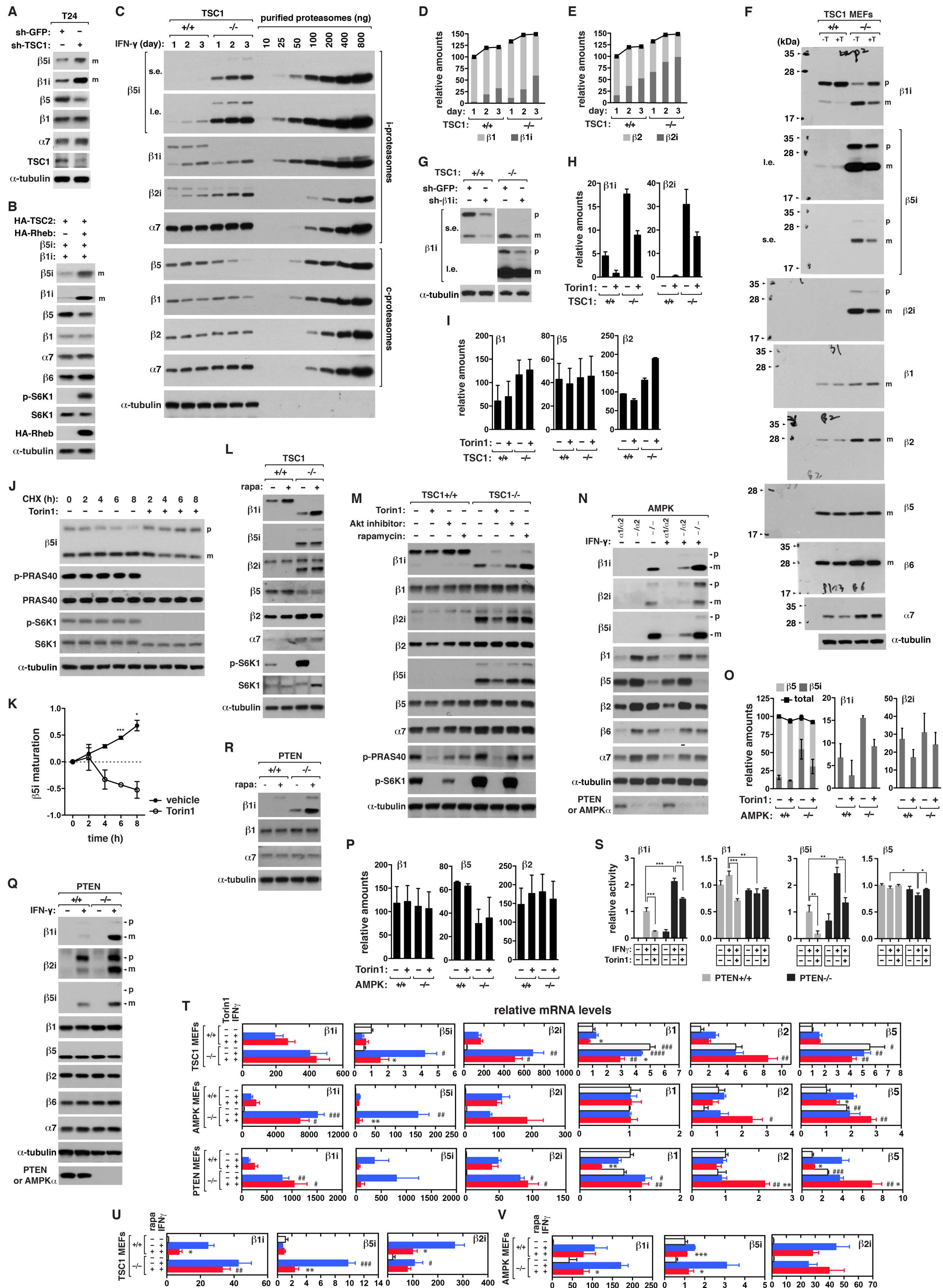

Figure S3, related to Figure 3. mTORC1 promotes the formation of i-proteasomes.

(**A**) TSC1 knockdown enhances the mature forms of β5i and β1i along with reduction of β5. T24 cells were stably transduced by shRNA for either GFP or TSC1. Cell lysate was analyzed for WB.

(**B**) Rheb overexpression increases the mature forms of β5i and β1i with reduction of β5. HA-tagged TSC2 and untagged β5i and β1i were transiently expressed with or without HA-Rheb in HEK293T cells. Cell lysate was analyzed for WB.

(**C**) Quantification of the amounts of β subunits affected by TSC1 deficiency. *TSC1*+/+ MEFs and *TSC1*-/- MEFs were treated with IFNγ (50 ng/ml) for the indicated days. The amounts of the indicated β subunits and α7 were analyzed by WB and compared with the amounts of subunits of purified i-proteasomes and c-proteasomes. α-tubulin was used as a loading control.

(**D, E**) Quantification of the amounts of β1 and β1i (D) and β2 and β2i (E) from the result of (C).

(**F**) The precursor and mature forms of the i-proteasome and c-proteasome β subunits along with the molecular size markers. Cell lysate was prepared and analyzed as described for Figure 3F. "l.e." and "s.e" indicate long exposure and shor exposure of films.

(**G**) Confirmation of the specificity of anti-β1i antibody. The antibody detects the precursor and mature forms of endogenous β1i. *TSC1* MEFs were stably transduced by the indicated shRNA, and treated with IFNγ (1 ng/ml) for 16 h. α-tubulin was used as a loading control.

(**H**) Quantification of the amounts of i-proteasomal β subunits in *TSC1* MEFs that are affected by Torin1. *TSC1* MEFs were treated with IFNγ (50 ng/ml) in the presence of Torin1 (250 nM) (+) or vehicle (-) for 16 h. The amounts of the indicated β subunits were analyzed by WB and compared with the amounts of subunits of i-proteasomes purified from mouse spleen. Values are means ± SD.

(**I**) Quantification of the amounts of c-proteasomal β subunits in *TSC1* MEFs that are affected by Torin1. The experiment was conducted as described in (H) except that c-proteasomes purified from mouse erythrocytes were used to quantify the amounts of c-proteasome subunits in MEFs. Values are means ± SD.

(**J**) Torin1 suppresses the maturation of β5i. An untagged version of β5i was transiently expressed in HEK293 cells. Two days post-transfection, cells were treated with cycloheximide (CHX, 5 μM) in the absence (-) or the presence (+) of Torin 1 (100 nM) for the indicated hours. Cell lystes were analyzed for the indicated proteins.

(**K**) Quantification of the result from (J). The reduced amount of β5i precursor and the increased amount of β5i mature form during the incubation were assessed by quantifying the difference of the band intensity at each time point from that at time 0 h. Means ± SD were obtained from the reduced amount of β5i precursor and the increased amount of mature β5i at each time point (*, $p<0.05$; ***, $p<0.001$ vs Torin1). The y-axis represents the extent of β5i maturation with 1 for the complete conversion of the precursor form at 0 h to the mature form.

(**L**) Rapamycin does not suppress i-proteasome formation. *TSC1* MEFs were treated with IFNγ (50 ng/ml) and rapamycin (100 nM) or vehicle (-) for 16 h.

(**M**) Rapamycin and Akt inhibitor do not suppress i-proteasome formation. *TSC1* MEFs were treated with IFNγ (50 ng/mL) in the presence or absence of Torin1 (250 nM), Akt inhibitor (2 μM), and/or rapamycin (100 nM) for 16 h.

(**N**) AMPK deficiency increases i-proteasome biogenesis. AMPK α1 and α2 intact MEFs, AMPK α1-deficient MEFs, and AMPK α1- and α2-deficient MEFs were treated with IFNγ (50 ng/ml) for 16 h.

(**O**) Quantification of the amounts of i-proteasome β subunits in *AMPK* MEFs. *AMPK*α1+/+;α2+/+ MEFs (+/+) and AMPKa1-/-;α2-/- MEFs (-/-) were treated with IFNγ (50 ng/ml) in the presence or absence of Torin1 (100 nM) for 16 h. I-proteasomes purified from mouse spleen were used as standard. Values are means ± SD.

(**P**) Quantification of the amounts of c-proteasomal β subunits in *AMPK* MEFs. The experiment was conducted as in (O) except that purified c-proteasome was used as standard. Values are means ± SD.

(**Q**) PTEN deficiency increases i-proteasome biogenesis in MEFs. PTEN MEFs were treated with IFNγ (50 ng/ml) for 16 h.

(**R**) Rapamycin enhances β1i maturation in *PTEN* MEFs. Cells were treated with IFNγ (50 ng/mL) for 16 h in the presence or absence of rapamycin (100 nM).

(**S**) PTEN deficiency enhances the proteolytic activities of i-proteasomes while decreasing the proteolytic activities of c-proteasomes. MEFs were treated with IFNγ (50 ng/mL) for 24 h. The i-proteosome-specific proteolytic activities in cell lysate were analyzed using Ac-PAL-AMC (β1i-specific) and Ac-ANW-AMC (β5i-specific) as substrates, and the proteolytic activities of c-proteasomes were analyzed using Ac-Nle-Pro-Nle-Asp-AMC (β1-specific) and Ac-WLA-AMC (β5-specific) as substrates. Values are means ± SD (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; n=3).
(**T**) Reverse transcriptase quantitative PCR (qPCR) analysis of mRNA levels of i-proteasomal and c-proteasomal β subunits in *TSC1*, *PTEN*, and *AMPK* MEFs. Cells were treated with IFNγ (+. 50 ng/mL) or vehicle (-) in the presence or absence of Torin1 (250 nM) for 16h. Values are means ± SEM ($^{\#}$, p<0.05; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$; $^{\#\#\#\#}$, $p<0.0001$ between +/+ and -/- cells; *, p<0.05; **, $p<0.01$ vs Torin1-untreated cells; n=3)
(**U, V**) Effects of rapamycin on mRNA levels of i-proteasomal β subunits in *TSC1* (U) and *AMPK* MEFs (V). Cells were treated with IFNγ (50 ng/mL) in the presence or absence of rapamycin (100 nM) for 16 h. Values are means ± SEM ($^{\#}$, p<0.05; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$ between +/+ and -/- cells; *, p<0.05; **, $p<0.01$; ***, $p<0.001$ vs rapamycin-untreated cells; n=3).

Figure S4, related to Figure 4

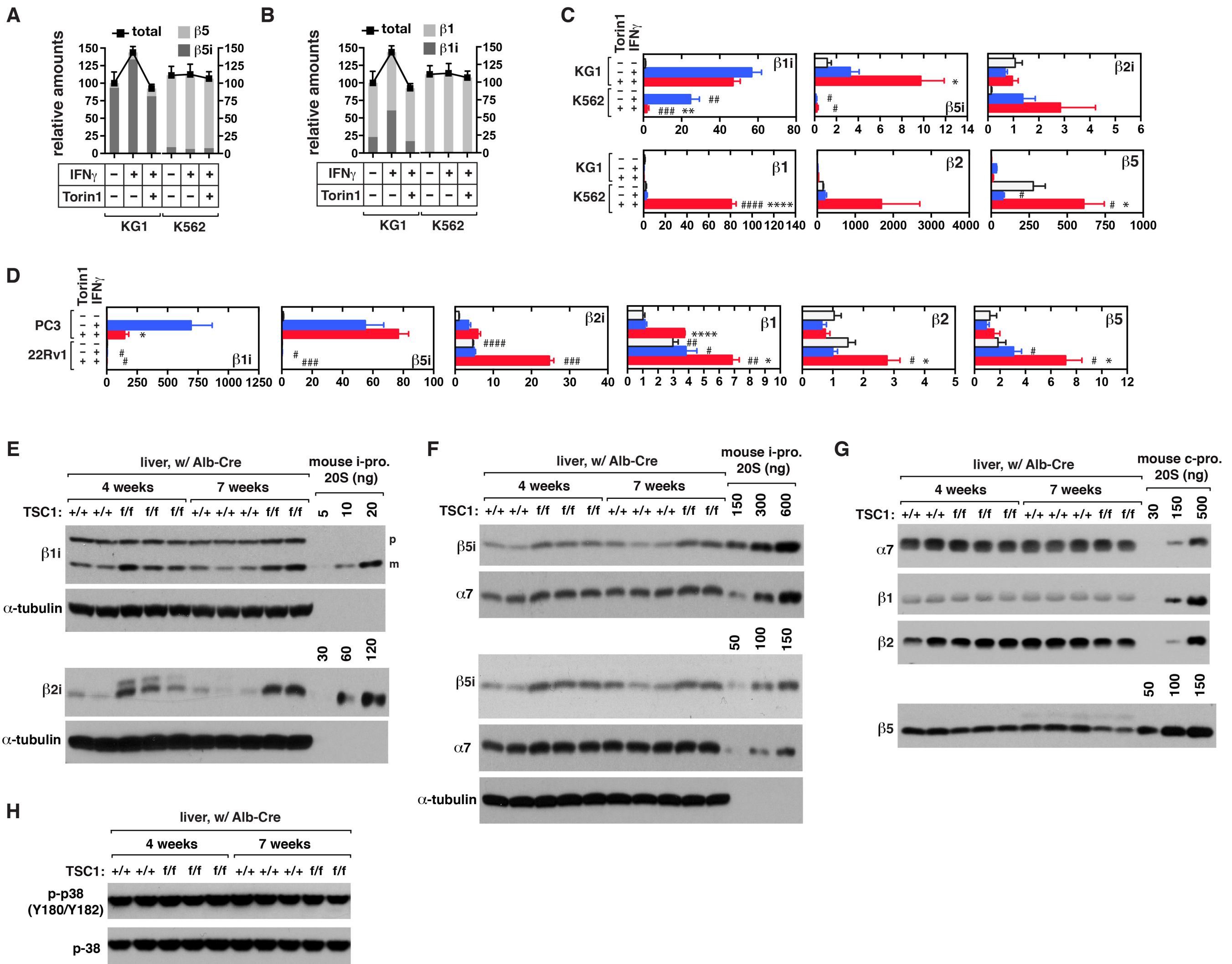

Figure S4, related to Figure 4. mTORC1 promotes i-proteasome formation in cancers, immortalized cells and in vivo.

(**A**) Quantitative analysis of the amounts of β5 and β5i in KG1 and K562 cells. The cells were treated with IFNγ (50 ng/ml) in the presence or the absence of Torin1 (250 nM) for 16 h. I-proteasomes and c-proteasomes purified from human peripheral blood mononuclear cells and erythrocytes, respectively, were used as standard. Values are means ± SD.

(**B**) Quantitative analysis of the amounts of β1 and β1i in KG1 and K562 cells. Values are means ± SD.

(**C**) qPCR analysis of mRNA levels of i-proteasomal and c-proteasomal β subunits in KG1 and K562 cells. Cells were treated with IFNγ (50 ng/mL) in the presence or absence of Torin1 (250 nM) for 16 h. Values are means ± SEM (#, p<0.05; ##, *p*<0.01; ###, *p*<0.001; ####, *p*<0.0001 between +/+ and -/- cells; *, p<0.05; **, *p*<0.01; ****, *p*<0.0001 vs Torin1-untreated cells; n=3).

(**D**) qPCR analysis of mRNA levels of i-proteasomal and c-proteasomal β subunits in PC3 and 22Rv1 cells. IFNγ and Torin1 were treated as described in (C). Values are means ± SEM (#, p<0.05; ##, *p*<0.01; ###, *p*<0.001; ####, *p*<0.0001 between +/+ and -/- cells; *, p<0.05; ****, *p*<0.0001 vs Torin1-untreated cells; n=3).

(**E-G**) Quantitative analysis of proteasomal α and β subunits in the mouse liver tissue. Male mice in the same cohorts were used. The amounts of the indicated subunits were analyzed by WB and compared with the amounts of subunits of purified proteasomes. α-tubulin is a loading control.

(**H**) TSC1 deficiency does not alter the phosphorylation of p38 at Y180/Y182 in the liver tissue.

Figure S5, related to Figure 5

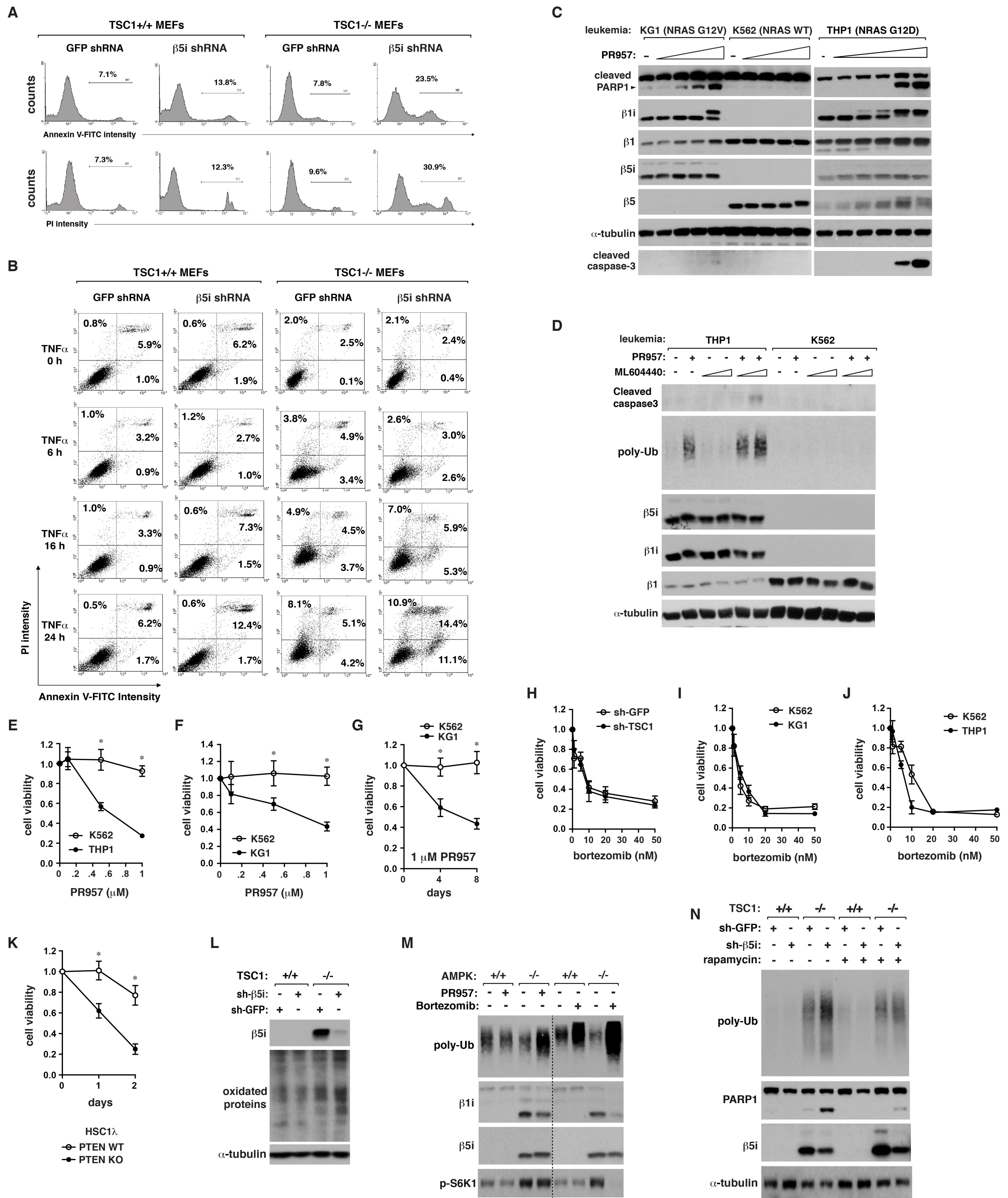

Figure S5, related to Figure 5. I-proteasomes are important for viability of mTORC1 hyperactive cells.

(**A**) β5i knockdown induces apoptosis to a greater extent in *TSC1*-/- MEFs compared to *TSC1*+/+ MEFs. *TSC1* MEFs were stably transduced by the indicated shRNA. Cells were treated with TNFα (20 ng/mL) for 24 h, stained with Annexin V-conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI), then analyzed by FACS.

(**B**) The experiment described in (A) was conducted for different durations of time (0, 6, 16, or 24 h) for the treatment of TNFα.

(**C**) I-proteasomal inhibition induces cleavages of PARP1 and caspase-3 in leukemia cells with *NRAS* mutation. KG1 and K562 cells were treated with PR957 at 0, 50, 100, 500, 1000 nM for 24 h, and THP1 cells were treated with PR957 at 0, 10, 50, 100, 500, 1000 nM for 16 h. The induction of apoptosis was confirmed by blotting cleaved PARP1 and caspase-3.

(**D**) Co-treatment of THP1 cells with β1i and β5i inhibitors highly induces accumulation of poly-Ub proteins and apoptosis. The leukemia cells were treated with PR957 (100 nM) and/or ML604440 (100 nM or 500 nM) for 24 h. Poly-Ub proteins were monitored by WB.

(**E, F**) PR957 induces leukemia cell death in a manner dependent upon the status of *NRAS* mutation. The indicated leukemia cells were treated with PR957 at the indicated concentrations for 48 h (E) or 8 days (F). Cell viability was assessed by MTT assay. Values are means ± SD (*, $p<0.05$; n=3).

(**G**) The indicated leukemia cells were treated with PR957 (1 μM) for 4 or 8 days. Values are means ± SD (*, $p<0.05$; n=3).

(**H-J**) Bortezomib induces cell death regardless of the status of *TSC1* and *NRAS* mutation. T24 cells stably transduced by the indicated shRNA (H) and the indicated leukemia cells (I, J) were treated with bortezomib at different concentrations for 48 h (H) or 4 days (I, J). Values are means ± SD.

(**K**) Biallelic deletion of *PTEN* in immortalized human schwann cells λ (HSC1λ) cells sensitizes the cells to i-proteasomal inhibition for viability. Cells were treated with PR957 (800 nM) for the indicated time. Values are means ± SD (*, $p<0.05$; n=3).

(**L**) Knockdown of β5i in TSC1-deficient MEFs induces accumulation of oxidized proteins. *TSC1* MEFs stably transduced with sh-GFP or sh-β5i were analyzed for the amounts of oxidized proteins using Oxyblot protein oxidation detection kit from Millipore (Billerica, MA).

(**M**) PR957, but not bortezomib, induces accumulation of poly-Ub proteins in a manner dependent upon AMPK status. *AMPK*α1+/+;α2+/+ MEFs (+/+) and *AMPK*α1-/-;α2-/- MEFs (-/-) were treated with of IFN-γ (50 ng/mL) for 72 h, then with PR957 (100 nM) or bortzomib (10 nM) for additional 24 h.

(**N**) Rapamycin suppresses accumulation of poly-Ub proteins in TSC1-deficient, β5i-silenced MEFs. *TSC1* MEFs transduced by the indicated shRNAs were treated with TNF-α (20 ng/ml) and/or rapamycin (100 nM) for 16 h. Rapamycin was added 3 h before TNF-α was added.

Figure S6, related to Figure 6

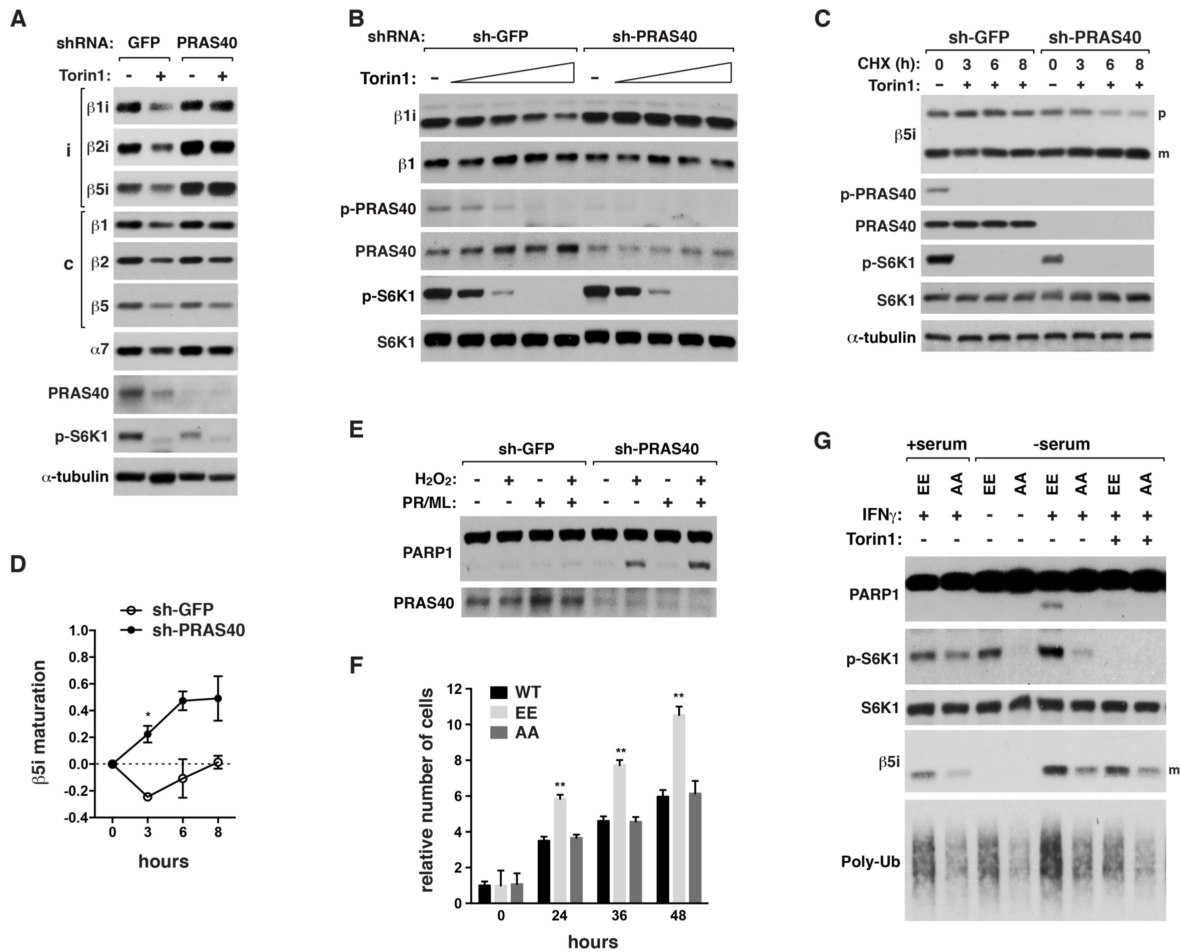

Figure S6, related to Figure 6. mTORC1 regulation of i-proteasome formation depends on PRAS40 and is important for cell survival against stress.

(**A**) PRAS40 knockdown made the maturation of i-proteasomal β subunits less responsive to Torin1 in HCT116 cells. HCT116 cells stably transduced by shRNA were treated with IFN-γ (50 ng/ml) for 24 h then with Torin1 (+, 250 nM) or vehicle (-) for additonal 48 h.

(**B**) PRAS40 knockdown made the β1i maturation less sensitive to Torin1 in KG1 cells. KG1 cells stably transduced by the indicated shRNA were treated with IFN-γ (50 ng/mL) and Torin 1 at 0, 20, 50, 100, 250 nM for 7 h.

(**C**) PRAS40 knockdown desensitizes the suppressive effect of Torin1 on β5i maturation. An untagged version of β5i was transiently expressed in shRNA-transduced HEK293 cells. Two days post-transfection, cells were treated with cycloheximide (5 μM) in the absence (-) or the presence (+) of Torin 1 (100 nM) for the indicated hours. Cell lystes were analyzed for the indicated proteins.

(**D**) Quantitative analysis of the result from (C). The reduced amount of β5i precursor and the increased amount of β5i mature form during the incubation were assessed by quantifying the difference of the band intensity at each time point from that at time 0 h. Means and SD were obtained from the reduced amount and the increased amount at each time point (*, $p<0.05$, sh-GFP vs sh-PRAS40).

(**E**) PRAS40 is important to protect cells against oxidative stress. KG1 cells stably transduced by sh-GFP or sh-PRAS40 were treated with H_2O_2 (5 μM) or vehicle in the absence (-) or presence (+) of PR957 (PR, 100 nM) and ML604440 (ML, 500 nM) for 16 h.

(**F**) PRAS40 EE mutation enhances cell proliferation. WT-, AA-, and EE-reconstituted HCT116 cells were cultured in 96 well plates. MTT assay was conducted at the indicated time points. Y-axis is relative number of cells in comparison to those at day 0. Values are represented as means ± SD (**, $p<0.01$ vs WT and AA, n=3).

(**G**) EE mutation induces apoptosis in serum-starved cells in a manner dependent upon mTORC1 activity. PRAS40 AA or EE-reconstituted HCT116 cells were treated with IFN-γ (50 ng/mL) for 24 h then starved of serum for additional 24 h in the presence or absence of Torin1 (250 nM).

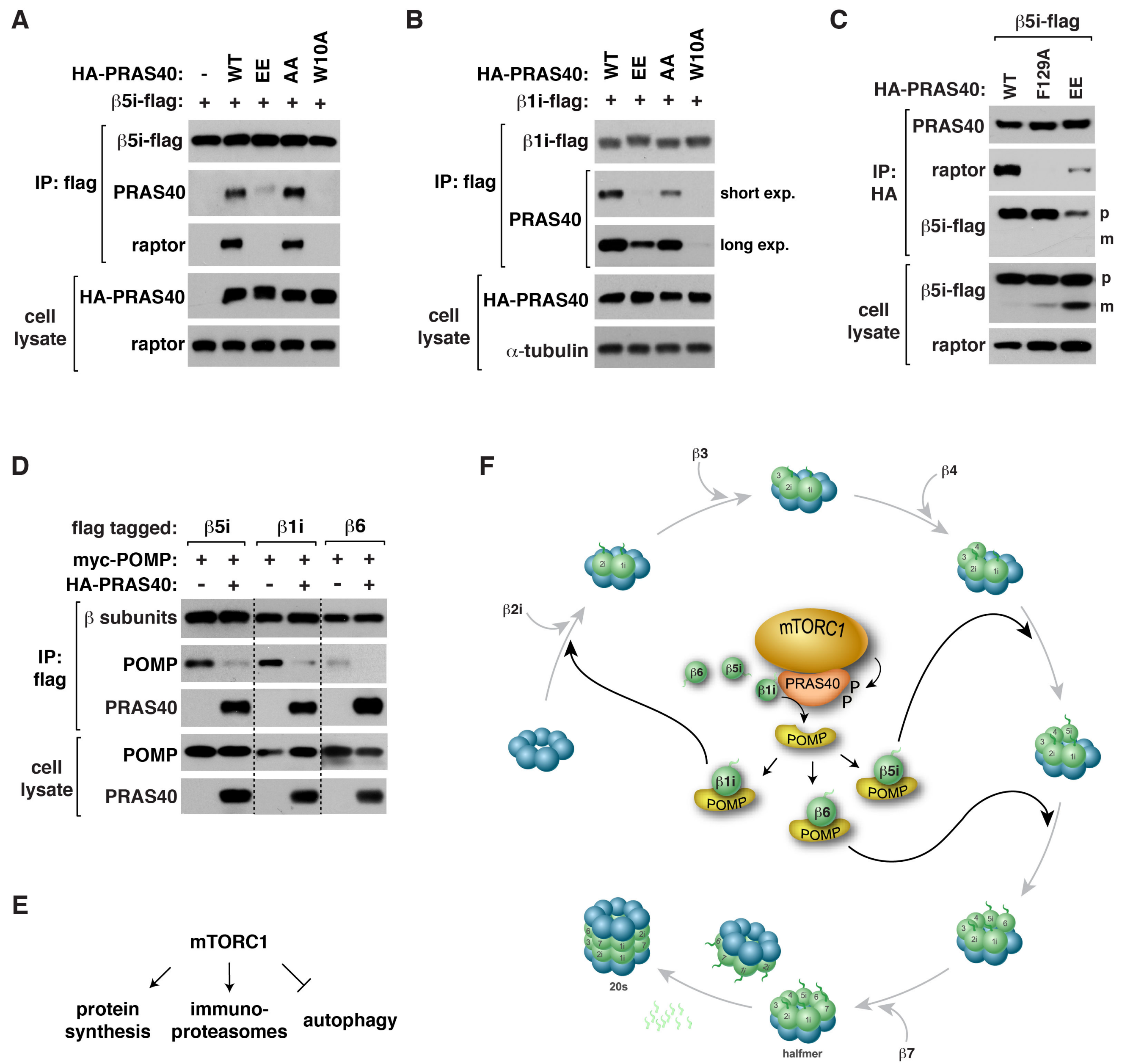

Figure S7, related to Figure 7. mTORC1 facilitates de novo biogenesis of i-proteasomes by phosphorylating PRAS40.

(**A**) PRAS40 EE mutation abolishes the interaction between PRAS40 and β5i. β5i-flag was transiently expressed alone (-) or together with PRAS40 constructs in HEK293T cells.

(**B**) PRAS40 EE mutation drastically reduces binding of PRAS40 to β1i. β1i-flag was coexpressed with myc-PRAS40 constructs in HEK293T cells.

(**C**) PRAS40 F129A mutation, which disrupts the PRAS40-raptor interaction, does not have any drastic effect on the PRAS40-β5i interaction and β5i maturation. HA-PRAS40 construct was expressed with β5i-flag in HEK293T cells. β5i and raptor recovered with HA-PRAS40 were analyzed by WB.

(**D**) PRAS40 inhibits the interaction between i-proteasomal β subunits and POMP. Flag-tagged β subunits and myc-tagged POMP were expressed with or without HA-PRAS40 in HEK293T cells. The amount of POMP recovered with flag IPs was analyzed by WB.

(**E**) mTORC1 regulates proteostasis through three downstream pathways.

(**F**) mTORC1 promotes the assembly of i-proteasome β subunit precursors for the formation of the i-proteasome 20S core.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

Antibodies for S6K1 (9202), phospho-S6K1 Thr389 (9205), AMPK (2532), cleaved caspase-3 (9661), PARP1 (9542), phospho-PRAS40 S183 (5936), PTEN (9552), and TSC1 (6935) were from Cell Signaling Technology (Danvers, MA). Antibodies for POMP (sc-271414), mTOR (sc-1549), GAPDH (sc-25778), α-tubulin (sc-12462), and TSC2 (sc-893) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for β5 (PW8895), mouse β5i (PW8200), α7 (PW8110), mono and poly ubiquitinylated conjugates (PW8810), PRAS40 (ab133584), β1 (ab3331), β2i (ab94623), β2 (ab22650), and human β5i (ab119959) were from AbCam (Cambridge, MA). Antibodies for mouse (ab3328) and human β1i (PA1-1960) were obtained from AbCam and ThermoFisher Scientific (Waltham, MA), respectively. Flag M2 affinity gel and anti-flag antibody (F3165) were from Sigma (St. Louis, MO). Anti-hemagglutinin (HA) antibody (HA.11) and anti-Myc 9E10 antibody were from Covance (Berkeley, CA) and EMD Biosciences (San Diego, CA), respectively. Anti-GST (27457701) was from GE Healthcare Life Sciences (Little Chalfont, UK). Polyclonal antibodies for human β6 were produced from rabbits using GST-fused-full length β6 purified from E. coli as antigen by the Yenzym custom antibody service (South San Francisco, CA). Torin1 was obtained from Tocris Bioscience (Ellisville, MO). Purified human i20S (E-370) and 20S (E-360) and mouse i20S (E-376) and 20S (E-355) were from Boston Biochem (Cambridge, MA). Akt inhibitor VIII (CD0223) was from Chemdea (Ridgewood, NJ). Bortezomib (J60378) was from Alfa Aesar (Ward Hill, MA). Human TNFα (210-TA-010), mouse TNFα (410-MT), human IFNγ (285-IF), and mouse IFNγ (485-MI-100) were purchased from R&D systems (Minneapolis, MN). Oxyblot protein oxidation detection kit was from Millipore (Billerica, MA). The fluorogenic peptide substrates for β1, β1i, β5i were synthesized by LifeTein LLC (South Plainfield, NJ), and the fluorogenic peptide substrate for β5 was purchased from R&D systems. All other chemicals were purchased from Sigma unless indicated otherwise. PR-957 (ONX-0914) was synthesized according to procedures previously reported (Muchamuel et al., 2009; Sharma et al., 2012; Shenk et al., 2007). ML604440 (Basler et al., 2012) was prepared following known synthetic procedures (Olhava and Danca, 2009).

Yeast two-hybrid screen

Human PRAS40 full-length cDNA was cloned into pDBTrp vector (Tucker et al., 2009). The bait plasmid was introduced into AH109 yeast cells using LiAc, and the transformed cells were selected on Trp- media. AH109 cells transformed with pDBTrp-PRAS40 plasmid were grown overnight in 50 ml Trp- dropout media. Cells were diluted to OD600 of 0.23 in 150 ml of Trp- media and cultured for several more hours until OD600 reached 0.7. Cells were pelleted, rinsed in ddH_2O, washed in 2.5 ml of TE/LiAc (550 µl 10xTE; 550 µl 1 M LiAc; 3.9 ml dH_2O), and resuspended in 2 ml TE/LiAc. The prepared AH109 cells were transformed with a human fetal brain cDNA library (Clontech, Mountain View, CA) using LiAc/PEG method. We used approximately 80 µg of the cDNA library. The total titer was 1.5 million colonies. After a heat shock at 42°C, the transformed cells were plated in 0.9% NaCl solution onto 30 plates (150 mm) of Trp-/Leu-/His-/3 mM 3AT media and incubated at 30°C for 7-10 days. LacZ assays were conducted by colony lift procedure for primary isolates by re-streaking the isolates on Trp-/Leu-/His-/3 mM 3AT plates. Briefly, colonies were lifted onto a nitrocellulose membrane, frozen in liquid nitrogen and thawed for cell lysis. The membrane was placed on a filter paper containing Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$-7H_2O, 39 mM β-mercaptoethanol, 1 mg/ml Xgal, pH 7) and incubated at 37˚C for 30 min for 2 h. Positive colonies that showed a color change in LacZ assays were picked for colony PCR. Alternatively, DNA was isolated from yeast and transformed into bacteria for miniprep analysis and sequencing. For PCR, cells were resuspended in 20 mM NaOH and boiled 15 minutes. Five microliter of each PCR product was treated with 2 µl EXO-SAP mix (89 µl H_2O, 1 µl exonuclease I (USB) and 10 µl shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) and incubated at 37˚C for 15 minutes, followed by inactivation at 80˚C for 15 minutes, before sequencing. Plasmids containing Gal4AD-cDNA library clones were recovered by transforming DNA into *E. coli* MH4 cells (*leuB*2, D*(lac)X74, galU, galK, hsr*2, *hsm*1, *strA*). Transformants were plated on M9 minimal medium lacking leucine but containing ampicillin. Plasmid DNA was isolated from bacteria and digested to confirm insert size. After sequencing, plasmid DNA was transformed into yeast strain AH109 in

combination with the original bait plasmid. Transformants were selected for growth on trp-/leu- plates, and streaked onto trp-/leu-/his-/3 mM 3-AT plates to test for growth.

Plasmids and DNA construction

Human and mouse proteasomal β subunit cDNAs were obtained from GE Healthcare Life Sciences. The cDNAs were cloned into pRK5 expression vector by PCR amplification to express them with HA or myc tag at N-terminus. To express C-terminal flag-tagged proteins, we cloned the genes into pCSII-EF-MCS vector. The genes were also cloned into pGEX6P2 vector (GE Healthcare) to express them in E. coli as GST-tagged proteins. The mutant constructs for PRAS40 W10A, S183A/S221A, F129A, S183E/S221E were generated using a site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA). The primer sequences for the mutations are 5'-cgccccgaggagctggcggaggccgtggtgggg-3' and 5'-ccccaccacggcctccgccagctcctcggggcg-3' for W10A; 5'–cagcagtacgccaaggccctgcctgtgtctgtg-3' and 5'-cacagacacaggcagggccttggcgtactgctg-3' for S183A; 5'-ggaccgcatcgcggcggccatgcgcgcgctggtg-3' and 5'-caccagcgcgcgcatggccgccgcgatgcggtcc-3' for S221A; 5'-acacagcagtacgccaaggaactgcctgtgtctgtgccc-3' and 5'-gggcacagacacaggcagttccttggcgtactgctgtgt-3' for S183E; 5'-ctggaccgcatcgcggcggaaatgcgcgcgctggtgctg-3' and 5'-cagcaccagcgcgcgcatttccgccgcgatgcggtccag-3' for S221E; 5'-gataatggagggctcgctgtgatggatgaggac-3' and 5'-gtcctcatccatcacagcgagccctccattatc-3' for F129A. Plasmids for shRNAs in pLKO.1 vector were obtained from GE Healthcare Life Sciences. The target sequence for mouse PRAS40 shRNA is described in our previous work (Vander Haar et al., 2007). The target sequences for mouse β1i and β5i shRNAs are 5'-gacttgttagcgcatctcata-3' and 5'-gaccaggaaaggaatgttcaa-3', respectively, and the target sequence for human PRAS40 3'-UTR region is 5'-gccttcaatttacgttcttta-3'.

Cell culture and transfection

HEK293, HEK293T, MEF, HCT116, T24, HSC1λ cells were cultured in DMEM containing 10% fetal bovine serum. 3T3-L1 cells were cultured in DMEM containing 10% calf serum. PC3 and 22Rv1 cells were cultured in RPMI 1640 containing 10% fetal bovine serum. KG1, THP1, K562 cells were cultured in RPMI 1640 media containing 20% fetal bovine serum. Penicillin/streptomycin was included in the culture media, and cells were cultured at 37°C in 5% CO_2. For transient expression, cells were transfected with recombinant DNAs using Fugene 6 (Promega Life Sciences, Madison, WI) following the manufacturer's protocol. For HCT116 cells, X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics) was used for transfection. Cells were harvested two or three days after transfection for co-immunoprecipitation assay. The *PTEN* deletion clones of HSC1λ cells were obtained using the TALEN technique as described in our previous report (Moriarity et al., 2014; Rahrmann et al., 2013).

Co-immunoprecipitation and western blotting

For co-immunoprecipitation experiments, whole-cell extracts were prepared in lysis buffer (40 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, and EDTA-free protease inhibitors) containing either 0.3% Chaps or 1% Trition X-100 as described previously (Kim et al., 2002). To detect endogenous interaction between PRAS40 and β6, HEK293T cells almost confluent in a 10 cm plate were rinsed with leucine-free RPMI twice, and incubated in leucine-free RPMI medium containing 10% dialyzed FBS for 24 h. Cells were lysed with lysis buffer containing 1% Triton X-100 and preceeded for co-immunoprecipitation experiments. Immunoprecipitated proteins were washed four times with lysis buffer, run on Tris–glycine gels (Invitrogen, Carlsbad, CA), and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) for western blotting. Advansta WesternBright ECL HRP substrate kits (BioExpress, Kaysville, UT) were used for detection of proteins.

Lentiviral preparation, viral infection, and stable cell generation

To prepare lentivirus for knockdown experiments, HEK293FT cells (Invitrogen) were transduced by pLKO-shRNA vector and lentiviral packaging vectors pHR'8.2ΔR and pCMV-VSV-G using Fugene 6 (Promega Life Sciences). To prepare lentivirus for protein expression, HEK293FT cells were transduced by CSII-EF-MCS vector encoding myc-PRAS40 WT or mutant with packaging vectors ΔNRF-HIV-1-gag-pol and pCMV-VSV-G using Fugene 6. For viral infection, we followed the procedure described in our previous report (Vander Haar et al., 2007).

In vitro binding assay

Human PRAS40, either untagged or myc-tagged, was cloned into pGEX6P2 (GE Healthcare Life Sciences) and expressed as GST-tagged forms in ArcticExpress bacteria (Agilent Technologies) by induction with IPTG (0.5 mM) at 12.5˚C for 20 h. Expressed proteins were purified using glutathione-sepharose 4B beads. GST was cleaved off from the fusion proteins using PreScission protease (GE Healthcare Life Sciences). Flag-tagged proteasomal β precursors were cloned into pGEX6P2 and expressed in *Arctic Express* bacteria. Bacterial extract was used for co-immunoprecipitation assay. For co-expression of PRAS40 and β precursors, we cloned both genes in pRSFDuet-1 vector (EMD Millipore Chemicals, Billerica, MA). Proteins were expressed at 12.5˚C with IPTG (0.5 mM) for 20 h in ArcticExpress bacteria. Bacterial extract was used for co-immunoprecipitation assay.

Proteasomal activity assay

Proteasomal activities in cell extract were conducted using fluorogenic peptide substrates. Protein concentration was adjusted to 1 µg/µl, and 3 µl of the lysate was added to 97 µl of reaction buffer containing 20 mM HEPES, pH 7.4 and the respective substrate. The activities of β1i, β1, β5i, and β5 were assessed using Ac-PAL-AMC, Ac-Nle-Pro-Nle-Asp-AMC, Ac-ANW-AMC, and Ac-WLA-AMC, respectively (Basler et al., 2012; Blackburn et al., 2010; Huber et al., 2012; Kisselev et al., 2003; Miller et al., 2013). The peptide substrate concentration was 50 µM. The reactions were monitored with excitation at 380 nm and emission at 460 nm using a spectrofluorometer (Gemini microplate fluorometer, Molecular Devices, CA). The reaction was started by incubating cell extract with the peptide at 37˚C. The hydrolysis of peptide was monitored for 30 min and was analyzed by a software program provided by the manufacturer (SoftMax Pro, Molecular Devices, CA). To confirm the specificity of the activities of β1i and β5i, we analyzed the activities in the presence or absence of ML604440 (500 nM) and PR957 (500 nM), respectively. The inhibitor was premixed with cell lysate for 10 min before the reaction was started. The activity of the inhibitor-treated sample was subtracted from that of the inhibitor-untreated sample. The activities of β1i and β5i were presented relative to the background activities of the samples obtained from IFNγ-untreated TSC1+/+, AMPK+/+, and PTEN+/+ MEFs, in which β1i and β5i were not detected.

Reverse transcriptase quantitative polymerase chain reaction (qPCR) assays

Total RNAs were prepared from cells using TRIzol reagent (Invitrogen). The first-strand cDNA was generated using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) and incubated with iQ SYBR Green Supermix (Bio-Rad). PCR was performed using an iCycler Real Time PCR System or CFX96 Touch Real time PCR detection system (Bio-Rad). β-actin was used as control. Results were analyzed using Bio-Rad CFX manager program supplied with CFX96 Touch Real time PCR detection system. Primers used are 5'-accttactagctgctcgggg-3' and 5'-agcctgcgatgatgattccc-3' for human β1 (PSMB6); 5'-ccaccagttggaggcttctc-3' and 5'-ctagggctgcaccaatgtaa-3' for human β2 (PSMB7); 5'-cgtgttggagagaccgctac-3' and 5'-ggcagctgctacagagatgc-3' for human β5 (PSMB5); 5'-gagcaccaaccggggactta-3' and 5'-ttcctcccagggttccatat-3' for human β1i (PSMB9); 5'-atgctgaagccagccctgga-3' and 5'-tacctgaagagcgtctggcg-3' for human β2i (PSMB10); 5'-atggcgctactagatgtatg-3' and 5'-actgacagtctgctgcacag-3' for human β5i (PSMB8); 5'-tacctcatgaagatcctcacc-3' and 5'-tgtaacgcaactaagtcatagtccgc-3' for human actin (ACTB); 5'-ccgccttagctgttcgtcga-3' and 5'-cagcctgcaatgatgattcc-3' for mouse β1 (Psmb6); 5'-ataattgtcgcaggaatgct-3' and 5'-caaaactagggctgcaccaa-3' for mouse β2 (Psmb7); 5'-gatgccggtgaatcagcacg-3' and 5'-agcgagcagtttggaggctg-3' for mouse β5 (Psmb5); 5'-ggaagaagtccacaccggga-3' and 5'-ccctcccatggttccatata-3' for mouse β1i (Psmb9); 5'-atgctgaagcaggcagtgga-3' and 5'-taccggaaaagcgtctggcg-3' for mouse β2i (Psmb10); 5'-atggcgttactggatctgtg-3' and 5'-tacaacctgcactccttggc-3' for mouse β5i (Psmb8); 5'-cctaaggccaaccgtgaaaa-3' and 5'-gaggcatacagggacagcaca-3' for mouse actin (ACTA1).

Brain tumor induction

FVB mice were purchased from the Charles River Laboratories and setup into mating pairs to generate neonatal mice for all experiments. Mating pairs were monitored daily. Neonatal mice less than 2 days of

age were used for all studies. Creation of PT/CAGGS-NRASV12 was described previously (Carlson et al., 2005). PT2/C-Luc/PGK-SB100 was a kind gift from Dr. John Ohlfest. Creation of PT2/shP53/GFP4 and empty vector was described previously (Wiesner et al., 2009). Plasmids were purified using an endotoxin-free maxiprep kit (Qiagen, Hilden, Germany) and diluted in 0.1x TE buffer. Tumors were induced by PEI/plasmid DNA administration by intracranial injection into the right lateral cerebral ventricle of neonatal mice as described previously (Wiesner et al., 2009). One microgram of total plasmid DNA was administered with 0.14 µl *in vivo*-jetPEI (Polyplus, Illkirch, France) in an injection volume of 1 µl 5% sterile dextrose. 0.25 µg each of the following plasmids was injected: PT2/C-luc-PGK-SB100, PT/CAGGS-NRASV12, PT2/shp53/GFP4, and empty vector. Successful plasmid uptake and transient expression was confirmed by imaging luciferase 24 h after injection. Twenty microliter of firefly luciferin (Goldbio) diluted to 28.5 mg/ml in sterile PBS was administered to mice by interperitoneal injection. Ten minutes after luciferin administration, mice were placed in a 6 well dish and imaged with a 5 min exposure on an IVIS fluorescence imager. The exact ages of the mice in analysis were: FVB#216 - 76 days; FVB#222 - 84 days; FVB#223 - 112 days; FVB#227 - 48 days. Experimental animals were monitored daily for signs of tumor development. All mice were maintained in a specific pathogen-free facility. After sacrifice, the whole brain was carefully removed from the mouse and placed in cold PBS. The brain was sliced in half along the longitudinal fissure using a sterile razor blade and examined. Tumors were carefully removed from the brain using fine forceps, placed in fresh cold PBS, and frozen at -80°C in RNAlater (Sigma). Protein was extracted from tumor tissue using an RNA/proteins purification kit (Norgen BioTek Corp., Thorold, ON, Canada) and stored at -80°C. The experiments followed the University of Minnesota Animal Care and Use Committee guidelines.

Flow cytometric analysis
We used Annexin V-FITC apoptosis detection kit 1 from BD Biosciences (Frankline Lakes, NJ). Briefly, cells were exposed to TNF-α (20 ng/ml) for the indicated time. After the exposure, cells were washed with cold PBS buffer and then detached with 0.05% trypsin/EDTA. The detached cells were stained with Annexin V- conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 60 min on ice, and then analyzed using a FACS Caliber system (BD Biosciences) with CellQuest and WinMDI 2.9 softwares.

Cell viability assay
For adhesion cells, cells were plated with a density of 30% confluence in 6-well plate and cultured in 2 ml medium/well. For suspension cells, the cell density was 1×10^6 in 2 ml medium. The cellular toxicity was measured by MTT assay. Briefly, 100 µl of MTT solution was added into 2 ml medium, and cells were incubated for 1-3 h. Medium was removed and DMSO was added to solubilize MTT. 100 µl of the solubilized MTT was transferred into 96-well plate to determine absorbance at 595 nm using 1420 multilabel counter VICTOR^{3}V (Perkin Elmer, Waltham, MA).

Primary leukemia cell self-renewal assay
We used the Mll-AF9/NRASG12V murine model of AML that encodes a tetracycline repressible NRASG12V oncogene (Kim et al., 2009). Primary leukemia cells were obtained from the spleens of the mice before the leukemia has completely abated (Sachs et al., 2014). For colony-forming assays, 10000 primary NRAS*(*G12V)/Mll-AF9 leukemia cells were plated in Methocult^{TM} M3231 (StemCell Technologies, Vancouver, Canada) with 1 ng/ml of GM-CSF (Invitrogen), 100 U/ml Penicillin G, 100 µg/ml streptomycin, and one of the following chemicals: doxycycline (Clontech, 1 µg/ml), PR957 or DMSO vehicle. The number of colonies that developed was enumerated after 7 days in culture.

Cell viability assay for pancreatic cancer cells
MIAPaCa2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2.5% horse serum, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% Glutamax-1. Cells were plated in 96-well plates at 25×10^4 cells per ml and allowed to adhere overnight. After 24 h, PR957 was added at 3x dilutions from 100 µM in growth media. Plates were incubated for 72 h at 37°C in 5% CO_2 after which the medium was removed and MTT was added in RPMI phenol red free media. MTT was removed after 3 h, formazan crystals were solubilized with 200 µl of isopropanol, and plates were read on M5e

spectrophotometer (Molecular Devices) at 570 nm for formazan and 690 nm for background subtraction. EC50 values were calculated using GraphPad Prism 6 (GraphPad Software Inc.).

Statistical analysis
Western blot bands were analyzed by Image J software for quantification of the amounts of precursor and mature forms of proteasomal subunits. Outcomes requiring statistical analysis, such as gene expression results and proteasome activities, were summarized using the mean and either standard deviation (SD) or standard error of the mean (SEM). Statistical significance was determined by the Student t test using Prism 6 software (GraphPad Software Inc.).

SUPPLEMENTAL REFERENCES

Basler, M., Lauer, C., Moebius, J., Weber, R., Przybylski, M., Kisselev, A.F., Tsu, C., and Groettrup, M. (2012). Why the structure but not the activity of the immunoproteasome subunit low molecular mass polypeptide 2 rescues antigen presentation. J Immunol *189*, 1868-1877.

Blackburn, C., Gigstad, K.M., Hales, P., Garcia, K., Jones, M., Bruzzese, F.J., Barrett, C., Liu, J.X., Soucy, T.A., Sappal, D.S*., et al.* (2010). Characterization of a new series of non-covalent proteasome inhibitors with exquisite potency and selectivity for the 20S beta5-subunit. The Biochemical journal *430*, 461-476.

Carlson, C.M., Frandsen, J.L., Kirchhof, N., McIvor, R.S., and Largaespada, D.A. (2005). Somatic integration of an oncogene-harboring Sleeping Beauty transposon models liver tumor development in the mouse. Proc Natl Acad Sci U S A *102*, 17059-17064.

Huber, E.M., Basler, M., Schwab, R., Heinemeyer, W., Kirk, C.J., Groettrup, M., and Groll, M. (2012). Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. Cell *148*, 727-738.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Kim, W.I., Matise, I., Diers, M.D., and Largaespada, D.A. (2009). RAS oncogene suppression induces apoptosis followed by more differentiated and less myelosuppressive disease upon relapse of acute myeloid leukemia. Blood *113*, 1086-1096.

Kisselev, A.F., Garcia-Calvo, M., Overkleeft, H.S., Peterson, E., Pennington, M.W., Ploegh, H.L., Thornberry, N.A., and Goldberg, A.L. (2003). The caspase-like sites of proteasomes, their substrate specificity, new inhibitors and substrates, and allosteric interactions with the trypsin-like sites. J Biol Chem *278*, 35869-35877.

Miller, Z., Ao, L., Kim, K.B., and Lee, W. (2013). Inhibitors of the immunoproteasome: current status and future directions. Current pharmaceutical design *19*, 4140-4151.

Moriarity, B.S., Rahrmann, E.P., Beckmann, D.A., Conboy, C.B., Watson, A.L., Carlson, D.F., Olson, E.R., Hyland, K.A., Fahrenkrug, S.C., McIvor, R.S*., et al.* (2014). Simple and efficient methods for enrichment and isolation of endonuclease modified cells. PLoS One *9*, e96114.

Muchamuel, T., Basler, M., Aujay, M.A., Suzuki, E., Kalim, K.W., Lauer, C., Sylvain, C., Ring, E.R., Shields, J., Jiang, J*., et al.* (2009). A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. Nat Med *15*, 781-787.

Olhava, E.J., and Danca, M.D. (2009). Proteasome Inhibitors. US 2009/0099132 A1.

Rahrmann, E.P., Watson, A.L., Keng, V.W., Choi, K., Moriarity, B.S., Beckmann, D.A., Wolf, N.K., Sarver, A., Collins, M.H., Moertel, C.L.*, et al.* (2013). Forward genetic screen for malignant peripheral nerve sheath tumor formation identifies new genes and pathways driving tumorigenesis. Nature genetics *45*, 756-766.

Sachs, Z., LaRue, R.S., Nguyen, H.T., Sachs, K., Noble, K.E., Mohd Hassan, N.A., Diaz-Flores, E., Rathe, S.K., Sarver, A.L., Bendall, S.C.*, et al.* (2014). NRASG12V oncogene facilitates self-renewal in a murine model of acute myelogenous leukemia. Blood *124*, 3274-3283.

Sharma, L.K., Lee, N.R., Jang, E.R., Lei, B., Zhan, C.G., Lee, W., and Kim, K.B. (2012). Activity-based near-infrared fluorescent probe for LMP7: a chemical proteomics tool for the immunoproteasome in living cells. Chembiochem : a European journal of chemical biology *13*, 1899-1903.

Shenk, K.D., Parlati, F., Zhou, H.-J., Sylvain, C., Smyth, M.S., Bennett, M.K., and Laidig, G.J. (2007). Compounds for Enzyme Inhibition. US 2007/0293465 A1.

Tucker, C.L., Peteya, L.A., Pittman, A.M., and Zhong, J. (2009). A genetic test for yeast two-hybrid bait competency using RanBPM. Genetics *182*, 1377-1379.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol *9*, 316-323.

Wiesner, S.M., Decker, S.A., Larson, J.D., Ericson, K., Forster, C., Gallardo, J.L., Long, C., Demorest, Z.L., Zamora, E.A., Low, W.C.*, et al.* (2009). De novo induction of genetically engineered brain tumors in mice using plasmid DNA. Cancer research *69*, 431-439.