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Supplemental Information

Negative Regulation of the Stability and Tumor Suppressor Function of Fbw7 by the Pin1 Prolyl Isomerase

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Figure S1, related to Figure 1

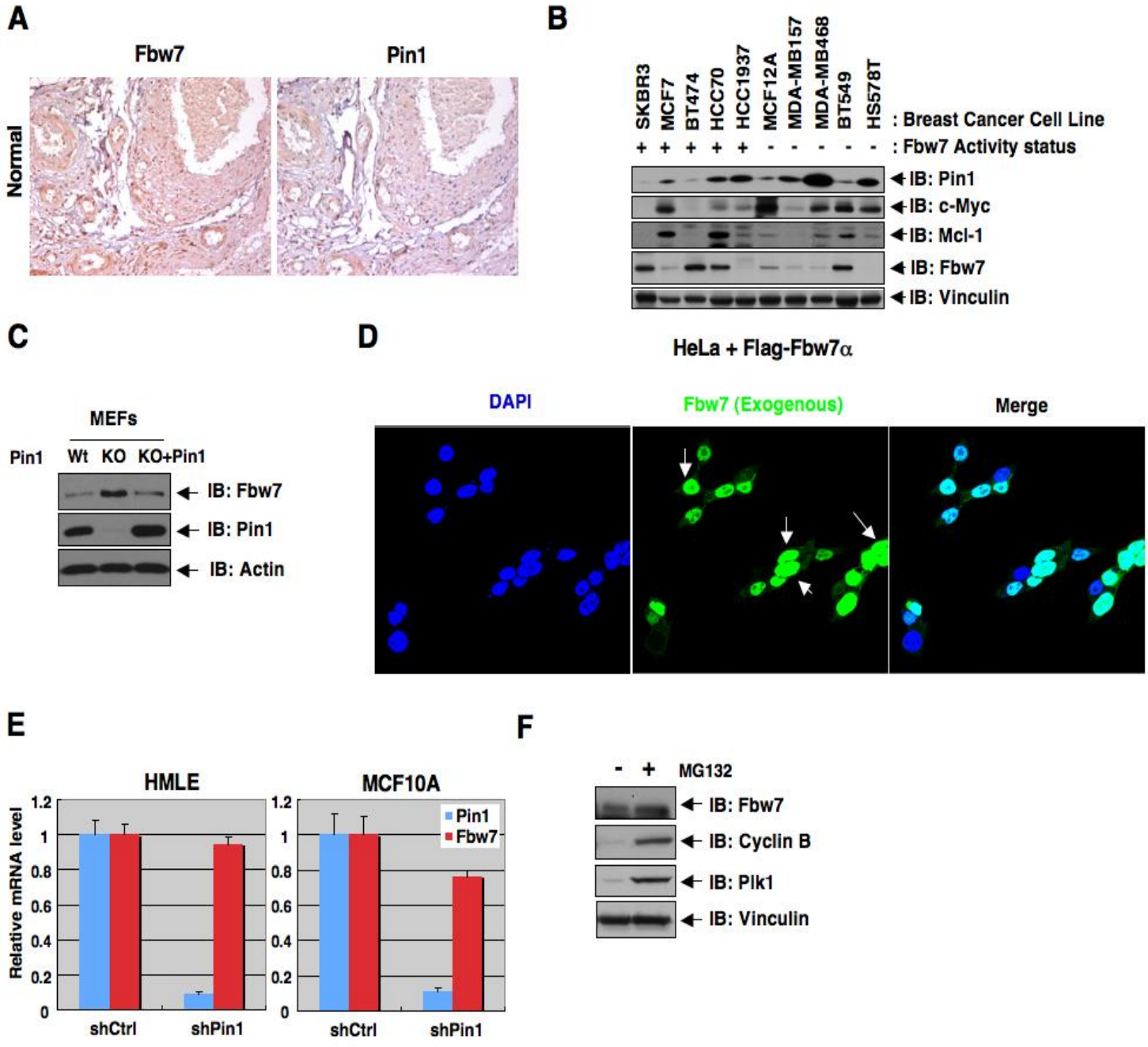

Figure S1: Negative regulation of Fbw7 protein abundance, but not Fbw7 mRNA levels by Pin1 (related to Figure 1).

A. Fbw7 abundance is inversely correlated with Pin1 expression in normal human colon tissue. Serial sections of tissue derived from normal human colon specimens were subjected to immunohistochemistry with anti-Pin1 and anti-Fbw7 antibodies, and visualized by DAB staining.

B. Immunoblot analysis of whole cell lysates derived from a panel of human breast cancer cell lines with the indicated antibodies.

C. Immunoblot analysis of whole cell lysates derived from WT or *Pin1*$^{-/-}$ MEFs. Where indicated, *Pin1*$^{-/-}$ MEFs were infected with a retrovirus encoding WT-Pin1 to generate a stable Pin1 expressing cell line before harvesting.

D. Anti-Fbw7 antibody used in Figures 1A and 1C was examined in cells ectopically expressing Fbw7. HeLa cells were transfected with Flag-Fbw7 and then subjected to immunostaining (green) with anti-Fbw7 antibody 30 hr post-transfection. DAPI staining is shown as blue.

E. Depletion of Pin1 does not affect Fbw7 mRNA levels. Both HMLE and MCF10A cells were infected with the indicated lentiviral shRNA constructs, followed by selection in 2 μg/ml puromycin for 72 hr to eliminate the non-infected cells before harvesting RNA for real time RT-PCR analysis. The error bars represent mean ± SD; n=3.

F. The abundance of endogenous Fbw7 is also regulated by the proteasomal degradation pathway. HCT116 cells were treated with MG132, followed by immunoblot analysis with the indicated antibodies.

Figure S2, related to Figure 2

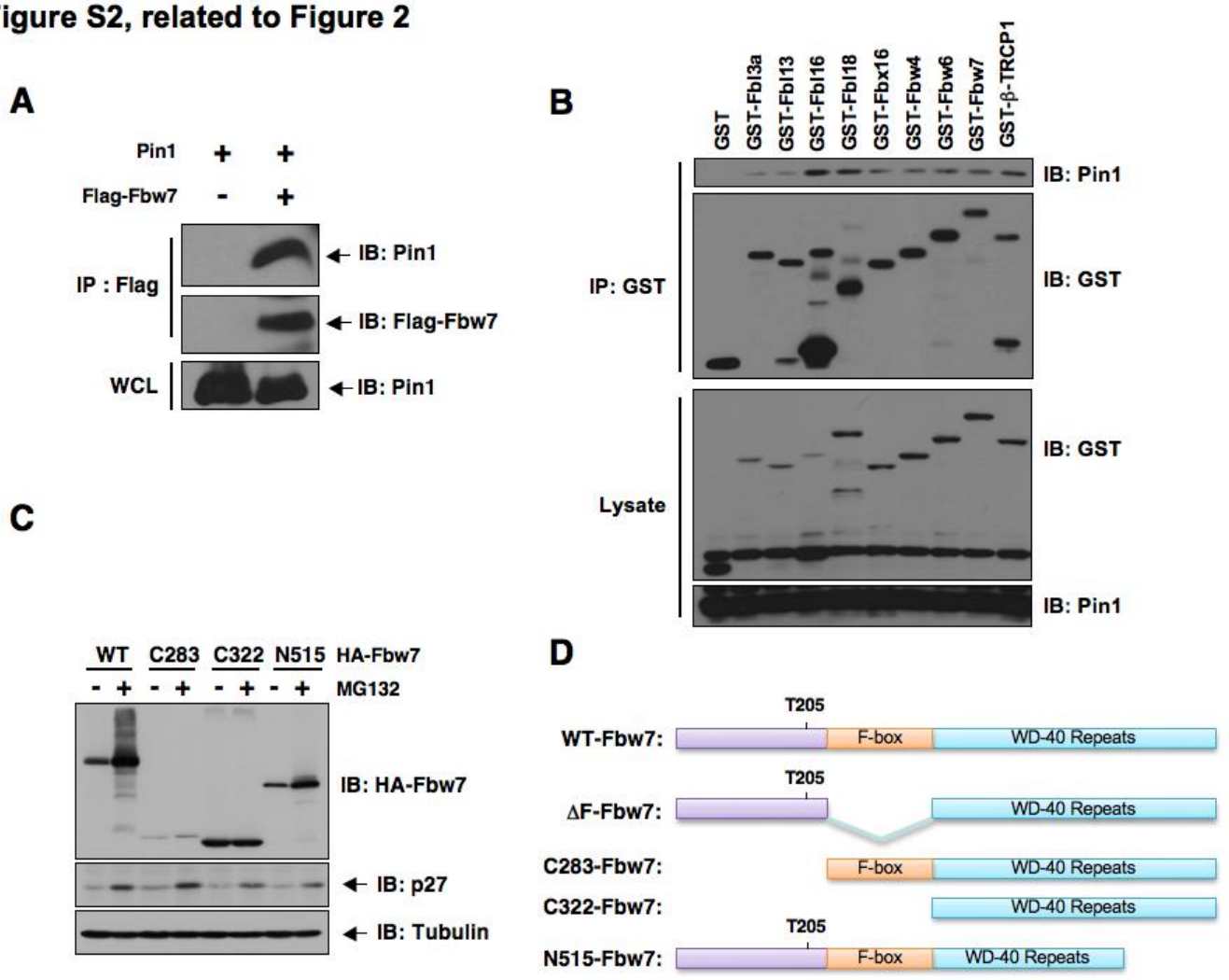

Figure S2: The N-terminus containing T205 site is critical for determining the sensitivity of Fbw7 to MG132 treatment (related to Figure 2).

A. Co-immunoprecipitation of ectopically expressed WT-Fbw7 and Pin1 *in vivo*. 293T cells were co-transfected with Flag-Fbw7 and HA-Pin1 constructs and then subjected to immunoprecipitation with anti-Flag, followed by immunoblotting with anti-Pin1 or anti-Flag antibodies.

B. Detected interaction between Pin1 and various indicated F-box proteins. Immunoblot analysis of whole cell lysates (WCL) and GST-immunoprecipitates derived from 293T cells co-transfected with the various indicated GST-tagged F-box proteins together with Myc-Pin1. Thirty hours post-transfection, cells were pretreated with 15 μM MG132 for 12 hr to block the proteasome pathway before harvesting.

C. Immunoblot (IB) analysis of whole cell lysates (WCL) derived from 293T cells transfected with the indicated HA-Fbw7 constructs. Thirty hours post-transfection, where indicated, cells were pretreated with 15 μM MG132 for 12 hr to block the proteasome pathway before harvesting.

D. Schematic representation of the various Fbw7 truncation mutants used in **C.**

Figure S3, related to Figure 3

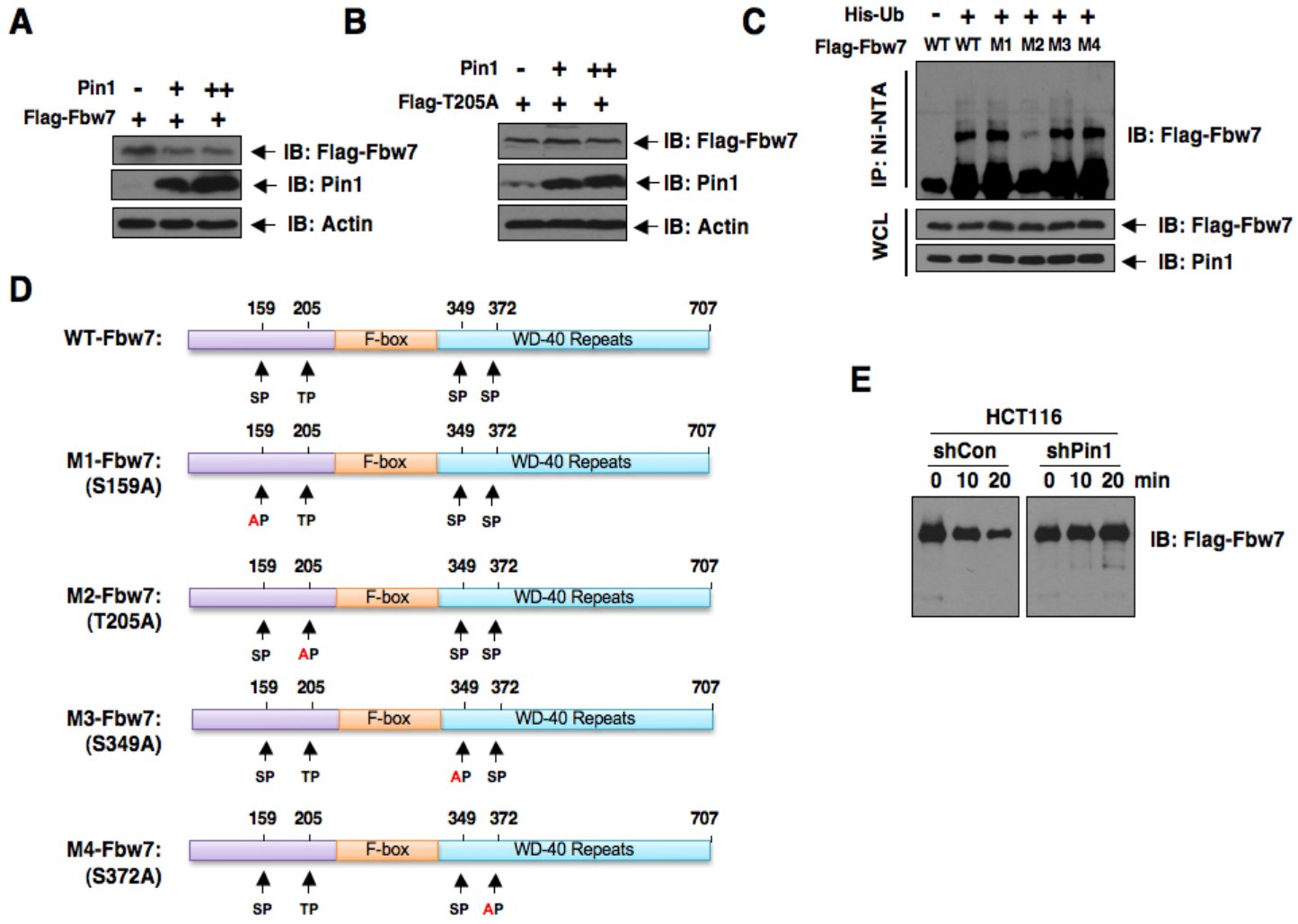

Figure S3: Pin1 negatively regulates Fbw7 stability by promoting its ubiquitination. (related to Figure 3).

A-B. WT, but not T205A, Fbw7 abundance is inhibited by wild-type Pin1. 293T cells were transfected with increasing amount of plasmid expressing WT-Pin1 together with the Flag-WT-Fbw7 (**A**) or Flag-T205A-Fbw7 (**B**) construct, followed by immunoblot analysis with the indicated antibodies.

C. The *in vivo* ubiquitination of the T205A mutant of Fbw7 is reduced compared to WT-Fbw7. HeLa cells were transfected with the indicated Flag-Fbw7 constructs and/or His-tagged ubiquitin or vector control as indicated, followed by lysis in a buffer containing 6M urea. Ubiquitin-conjugated proteins were captured with nickel-agarose beads and subjected to immunoblot analysis with anti-Flag antibody.

D. Schematic illustration of the various Pin1 mutants used in **C**.

E. HCT116 *Fbw7*$^{-/-}$ cells were infected with shPin1 (or with shGFP as a negative control) lentiviral vector and selected in 1 μg/ml puromycin for 72 hours to eliminate the non-infected cells. The resulting cell lines were transfected with a Flag-Fbw7 construct. 40 hours post-transfection, anti-Flag immunoprecipitation was performed to purify Fbw7 proteins, which were then used for trypsin digestion for the indicated time periods before subjected to immunoblot analysis with the indicated antibodies.

Figure S4, related to Figure 4

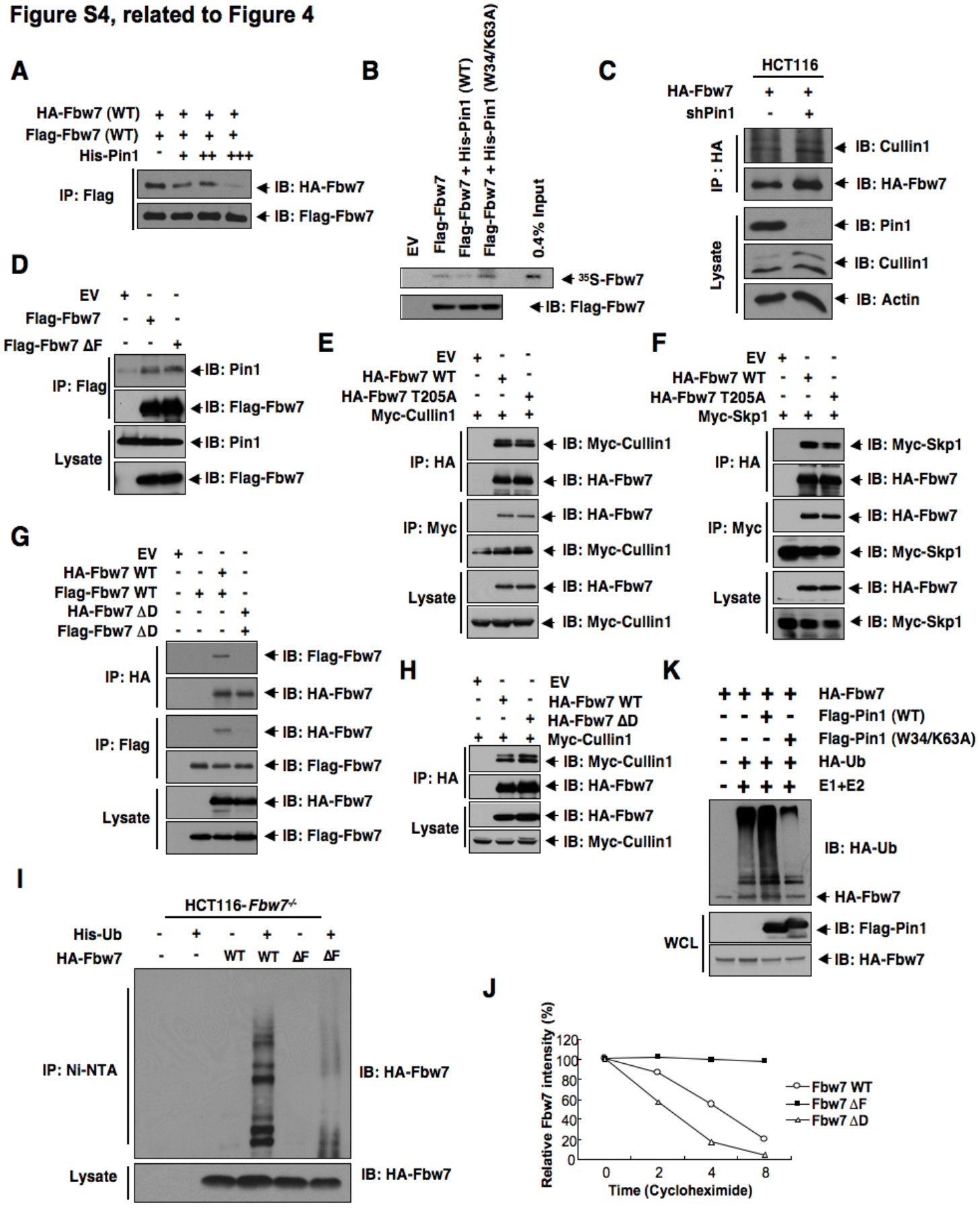

Figure S4, related to Figure 4

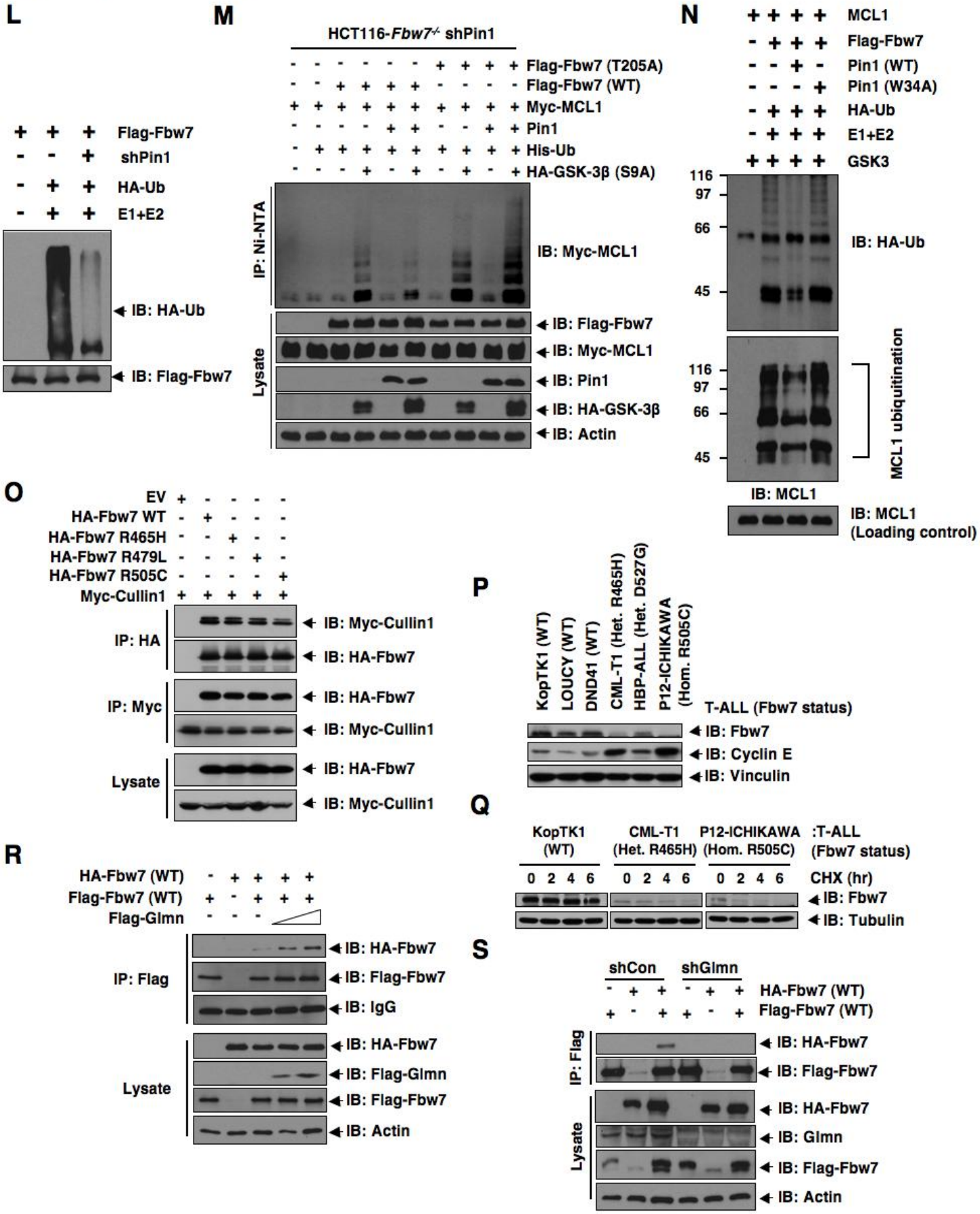

Figure S4. Pin1 promotes Fbw7 self-ubiquitination by inhibiting Fbw7 dimer formation (related to Figure 4).

A. HCT116 *Fbw7*$^{-/-}$ cells were transfected with both HA-Fbw7 and Flag-Fbw7 constructs. 40 hours post-transfection, whole cell lysates were harvested and incubated with increasing amounts (0, 0.3, 1 or 3 μM) of bacterially purified His-Pin1 before performing the anti-Flag immunoprecipitation. The recovered Fbw7 dimer was monitored by immunoblot analysis.

B. HCT116 cells were transfected with a Flag-Fbw7 construct. 40 hours post-transfection, whole cell lysates were harvested to affinity-purify Flag-Fbw7 with anti-Flag agarose-beads. The recovered Fbw7 immunoprecipitates were then incubated with ^{35}S-labeled Fbw7 in the presence of 3 μM of bacterially purified WT or W34A/K63A mutant Pin1. The recovered Fbw7 dimer was monitored by autoradiography.

C. Stable Pin1 shRNA infected HCT116 cells (with shGFP-infected HCT116 cells as a negative control) were infected with the lentiviral vector encoding for HA-Fbw7 and then selected with 100 μg/ml Hygromycin for 72 hr to eliminate the non-infected cells. The resulting cell lines were subjected to immunoprecipitation with anti-HA, followed by immunoblotting with the indicated antibodies.

D. 293T cells were transfected with the indicated Flag-Fbw7 constructs, followed by immunoprecipitation with Flag-agarose beads and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

E-F. 293T cells were transfected with the indicated HA-Fbw7 constructs together with Myc Cullin-1 (**E**) or Myc-Skp1 (**F**), followed by immunoprecipitation with anti-HA and anti-Myc antibodies, and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

G. HeLa cells were transfected with the indicated HA-Fbw7 or Flag-Fbw7 constructs (with empty vector as a negative control), followed by immunoprecipitation with anti-HA and anti-Flag antibodies, and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

H. 293T cells were transfected with the indicated HA-Fbw7 constructs together with Myc Cullin-1, followed by immunoprecipitation with anti-HA antibody, and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

I. Depletion of the F-box motif led to reduced *in vivo* Fbw7 ubiquitination. HCT116-*Fbw7*$^{-/-}$ cells were transfected with the indicated HA-Fbw7 and/or His-tagged ubiquitin or vector control as indicated, followed by immunoprecipitation with nickel-agarose beads and subjected to immunoblot analysis with anti-HA antibody.

J. A semi-quantification of the results presented in **Figure 4I** was performed using actin as a loading control and relative Fbw7 levels at time 0 set as 100%.

K. HCT116 cells were co-transfected with a HA-Fbw7 construct together with a plasmid encoding WT or a W34A/K63A mutant form of Pin1. 40 hours post-transfection, anti-HA immunoprecipitation was performed to affinity-purify the SCF^{Fbw7} complex. The affinity purified SCF^{Fbw7} complex was then incubated with recombinant E1, E2 and HA-Ubiquitin proteins at 30°C for 60 minutes. Fbw7 self-ubiquitination was monitored by immunoblot analysis with the indicated antibodies.

L. HCT116 *Fbw7*$^{-/-}$ or shPin1-HCT116 *Fbw7*$^{-/-}$ cells were transfected with a Flag-Fbw7 construct. 40 hours post-transfection, anti-Flag immunoprecipitation was performed to affinity-purify the SCF^{Fbw7} complex followed by elution with 3xFlag peptides. The affinity purified SCF^{Fbw7} complex was then incubated with recombinant E1, E2 and HA-Ubiquitin proteins at 30°C for 60 minutes. Fbw7 self-ubiquitination was monitored by immunoblot analysis with the indicated antibodies.

M. Expression of Pin1 led to reduced ability of WT-Fbw7, but not T205A-Fbw7 to promote *in vivo* Mcl-1 ubiquitination. HCT116 *Fbw7*$^{-/-}$ cells were infected with a shPin1 lentiviral construct followed by selection in 1 μg/ml puromycin containing medium to generate a stable Pin1 depleted cell line. Afterwards, this cell line was transfected with the indicated HA-Fbw7, Myc-MCL1, HA-GSK3, Pin1 and/or His-tagged ubiquitin or vector control as indicated, followed by immunoprecipitation with nickel-agarose beads and subjected to immunoblot analysis with anti-Myc antibody.

N. HCT116 *Fbw7*$^{-/-}$-shPin1 cells were transfected with a Flag-Fbw7 construct. 40 hours post-transfection, anti-Flag immunoprecipitation was performed to affinity-purify the SCF^{Fbw7} complex followed by elution with 3xFlag peptides. 0.3 μM of affinity purified SCF^{Fbw7} complex was then incubated with recombinant E1, E2 and HA-Ubiquitin proteins in the presence of 1 μM purified recombinant WT-Pin1 or W34A/K63A-mutant Pin1 together with GST-MCL1 that was pretreated with GSK3. *In vitro* MCL1 ubiquitination was monitored by immunoblot analysis with the indicated antibodies.

O. 293T cells were transfected with the indicated HA-Fbw7 constructs together with Myc-Cullin-1 (with empty vector as a negative control), followed by immunoprecipitation with anti-HA and anti-Myc antibodies, and then subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

P. Immunoblot analysis of a panel of T-ALL cell lines with the indicated antibodies.

Q. Endogenous Fbw7 stability in the indicated T-ALL cell lines was determined by using cycloheximide chase.

R. Stable shGlomulin-infected U2OS cells were transfected with Flag-Fbw7 and HA-Fbw7 constructs in the presence or absence of increasing amount of Flag-Glomulin (with empty vector as a negative control), followed by immunoprecipitation with Flag-agarose beads and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

S. Stable shGlomulin-infected U2OS cells (with shGFP-infected cells as negative controls) were transfected with Flag-Fbw7 and HA-Fbw7 constructs, followed by immunoprecipitation with Flag-agarose beads and subjected to immunoblot analysis with the indicated antibodies. 10 μM MG132 was added for 12 hr before harvesting the whole cell lystates.

Figure S5, related to Figure 5

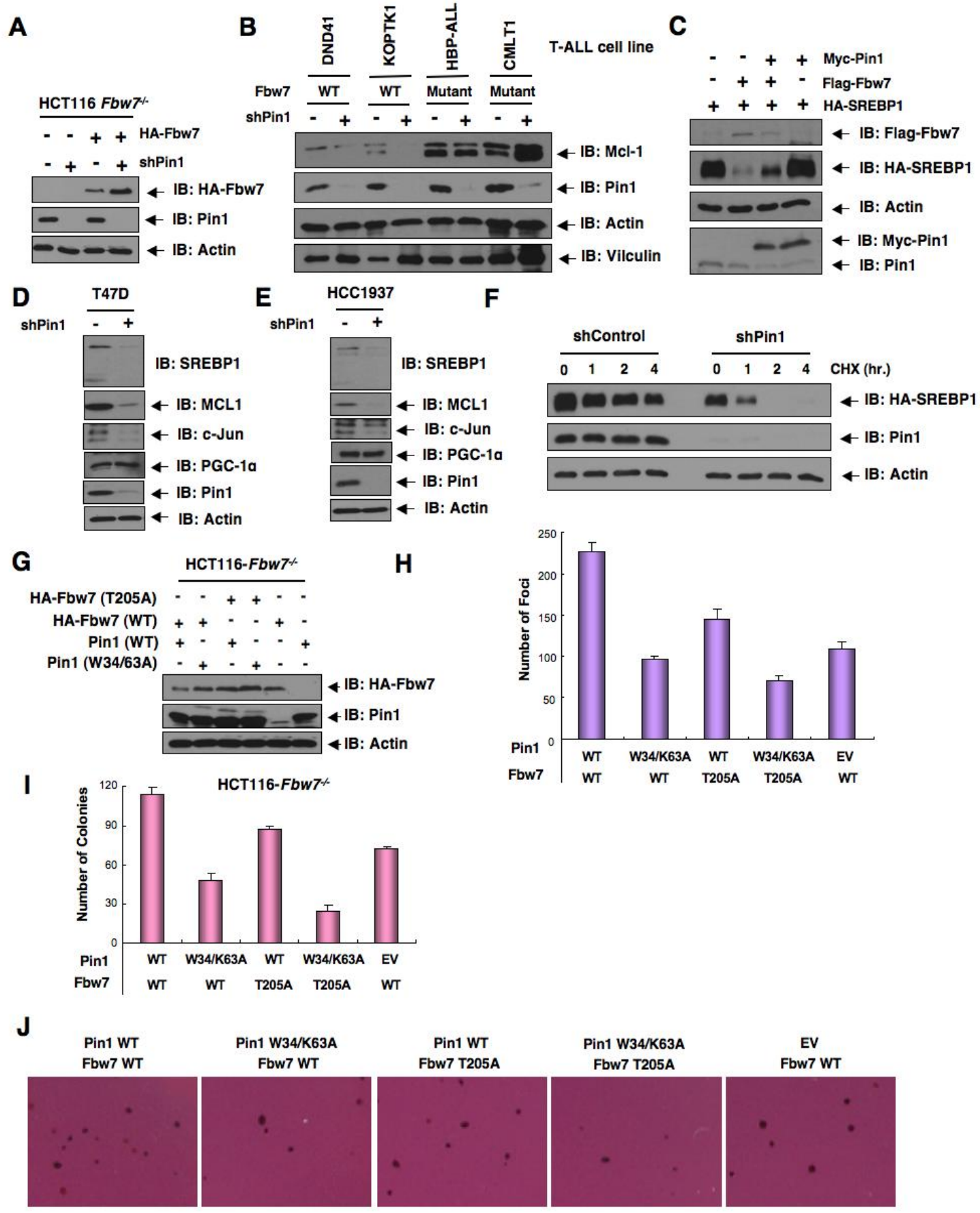

Figure S5. Pin1 negative reglates the tumor suppressor function of Fbw7 (related to Figure 5).

A. Immunoblot analysis of whole cell lysates derived from the various generated *Fbw7*$^{-/-}$ HCT116 cells, which were used to perform the functional assays in Figures **5A-G**.

B. Depletion of Pin1 leads to the reduction of the Fbw7 substrate Mcl-1 in T cell acute lymphoblastic leukemia (T-ALL) cells with WT-Fbw7, but not in T-ALL cells deficient in Fbw7 function. The indicated T-ALL cells were infected with shPin1 lentivirus (or shGFP as a negative control), followed by selection with 1 μg/ml puromycin, and then subjected to immunoblot analysis with the indicated antibodies.

C. The abundance of the Fbw7 substrate SREBP1 is regulated by Pin1. 293T cells were transfected with plasmids expressing HA-SREBP1 and Flag-Fbw7 in the presence or absence of WT Pin1, followed by immunoblot analysis with the indicated antibodies.

D-E. Immunoblot analysis of the whole cell lysates derived from T47D (**D**) or HCC1937 (**E**) cell lines infected with the indicated shRNA lentiviral constructs. Cells were selected in 1 μg/ml puromycin for 72 hours to eliminate the non-infected cells before harvesting.

F. The half-life of ectopically expressed HA-SREBP1 was measured in the indicated HCT116 cell lines by using cycloheximide chase.

G. Immunoblot analysis of whole cell lysates derived from the various generated *Fbw7*$^{-/-}$ HCT116 cells, which were used to perform the functional assays in **H-I.**

H-I. The various generated HCT116 *Fbw7*$^{-/-}$ cells were seeded on plastic plates (**H**) or in soft agar (**I**) for 2 or 3 weeks respectively, followed by crystal violet staining (**H**) or P-iodonitrotetrazolium violet staining (**I**). The error bars represent mean ± SD; n=3.

J. Representative images of the anchorage-independent colony growth of the various generated HCT116 *Fbw7*$^{-/-}$ cells after P-iodonitrotetrazolium violet staining in (**I**).

Supplemental Experimental Procedures

Plasmids

The N-terminal GST, Flag or HA tagged Pin1 wild-type and deletion mutants were described previously (Lee et al., 2011; Lu et al., 2002). The expression constructs for Flag- or HA- tagged wild type and various mutants of Fbw7 were also described previously (Inuzuka et al., 2011). The Fbw7 mutants where residues S156, S205, S349 or S372 were replaced by alanine, were generated by site-directed mutagenesis (Agilent) from wild type Flag-Fbw7 or HA-Fbw7. Flag-Glomulin mammalian expression construct and shGlomulin lentiviral construct were described previously (Tron et al., 2012). The shPin1 construct was described before (Lee et al., 2011; Lee et al., 2009; Ma et al., 2012).

GST Pulldown, Immunoprecipitation and Immunoblot Analysis

GST pulldown, immunoprecipitation, and immunoblotting analyses were performed as described previously (Inuzuka et al., 2011; Lee et al., 2011; Lu et al., 1999; Tun-Kyi et al., 2011). Briefly, relevant proteins were expressed in 293T, HCT116 *Fbw7*$^{-/-}$, or HeLa cells by transient transfection, followed by lysis in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride and 1 mM DTT. 10 μM MG132 was added for 12 hr to block the 26S proteasome before harvesting the whole cell lystates for the subsequent immunoprecipitation assays. The cellular supernatants were incubated with 1 μM GST (or the indicated GST fusion proteins), or the indicated antibodies for 1 hr at 4°C. Following incubation, 15 μl of glutathione agarose beads (for GST-pull down assays) or protein A agarose (for immunoprecipitation assays) were then added, followed by further incubation for 2 hr at 4°C. The precipitated proteins were washed 4-6 times in the same lysis buffer and subjected to immunoblotting analysis. Bacterially purified recombinant WT- or W34A, W34/K63A mutant forms of Pin1 were produced

as described previously (Lee et al., 2011; Ryo et al., 2003). To purify Fbw7 from mammalian cells, HCT116 *Fbw7*$^{-/-}$ cells are transfected with a HA-Fbw7 and/or a Flag-Fbw7 construct. 40 hours post-transfection, whole cell lysates were harvested for anti-HA or anti-Flag immunoprecipitation. 3xHA or 3xFlag peptides (from Sigma) were used to elute the immunoprecipitates for use in the subsequent *in vitro* biochemical experiments.

***In vitro* Ubiquitination Assay:**

The *in vitro* ubiquitination assays were performed as described previously (Koepp et al., 2001). Purified, recombinant GST-MCL1 proteins (Inuzuka et al., 2011) were incubated with purified SCF^{Fbw7} complexes (3 μg affinity-purified and eluted protein used in each reaction) in the presence of purified, recombinant active E1, E2 (UbcH5a and UbcH3), ATP and ubiquitin. The reactions were stopped by the addition of 2X SDS-PAGE sample buffer and the reaction products were resolved by SDS-PAGE and probed with the indicated antibodies. Where indicated, 1 μM of recombinant WT-Pin1, as described for other Pin1 substrates (Lu et al., 1999; Nakamura et al., 2012; Zhou et al., 2000) or W34/K63A-mutant Pin1 were preincubated with purified SCF^{Fbw7} complexes before the reaction. GST-MCL1 protein was pretreated with GSK3 purchased from New England Biolabs as described previously (Inuzuka et al., 2011) before use for the *in vitro* ubiquitination assays. Briefly, 5 μg of the indicated GST fusion proteins were incubated with purified active GSK3 in the presence of 200 μM cold ATP in the kinase reaction buffer for 20 min.

Real-time RT-PCR Analysis:

Total RNA was extracted using the Qiagen RNeasy mini kit, and the reverse transcription reaction was performed using the ABI Taqman Reverse Transcriptional Reagents (N808-0234). After mixing the generated cDNA templates with primers/probes and ABI Taqman Fast Universal PCR Master Mix (4352042), the real-time RT-PCR was performed with the ABI-7500 Fast Real-time PCR system. Human

Fbw7 (Hs00217794_m1), human Pin1 (Hs01598309_m1), and human GAPDH (Hs99999905_m1) primers were purchased from ABI.

Protein Stability Assay

For protein stability assays, cells were transfected stably or transiently with expression plasmids as indicated in the figure legends. Cycloheximide (100 μg/ml) was added to the media to block new protein synthesis. Cells were harvested at the indicated time points, and whole cell lysates were analyzed by immunoblot analysis with the indicated antibodies. The blots were scanned and semi-quantitated using the NIH image 1.6.2 software, as described previously (Lee et al., 2009). The results from at least three independent experiments are plotted and the protein levels at 0 h time point was set at 1.

Establishment of Stable Cell Lines

DLD1 *Fbw7*$^{+/+}$ (WT) and *Fbw7*$^{-/-}$ cells were infected with Pin1 shRNA or control shRNA lentiviruses, followed by selection with 2 μg/ml of puromycin. HCT116 *Fbw7*$^{+/+}$ and *Fbw7*$^{-/-}$ cells were infected with retrovirus encoding wild type HA-Fbw7 or control constructs and stable lines were selected using 100 μg/ml of Hygromycin, as described previously (Inuzuka et al., 2011). To deplete Pin1 in HCT116 *Fbw7*$^{+/+}$ and *Fbw7*$^{-/-}$ cells expressing HA-Fbw7, the Hygromycin selected cells were sequentially infected with Pin1 shRNA or control shRNA lentiviruses, followed by selection using 2 μg/ml of puromycin. Stable cell clones were checked for protein expression by immunoblot analysis with anti-HA and anti-Pin1 antibodies to confirm the expected protein expression. We maintained stable cell lines continuously in culture, splitting on every fourth day and seeding at the concentration of $6x10^5$ cells per 10 cm culture dish.

Supplemental References

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