Supporting Information

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SI Materials and Methods

Cell Culture, Plasmids, Antibodies, and Reagents. Human 293, HeLa, and H1299 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS, 50 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere as previously described (1, 2). MCF10A cells were grown in medium containing 1× penicillin/streptomycin, 45% (vol/vol) DMEM, 45% (vol/vol) F-12 Ham's, 5% (vol/vol) horse serum, 2.5 mM L-glutamine, 20 ng/mL EGF, 10 μg/mL insulin, 500 ng/mL hydrocortisone, and 100 ng/mL cholera toxin (3). Culture media for other breast cancer cell lines were as follows: SKBR3, MDA-MB-231, MDA-MB-453, MDA-MB-468, and MDA-MB-436 [DMEM with 10% (vol/vol) FBS]; MCF7 [45% (vol/vol) DMEM, 45% (vol/vol) F-12 Ham's, and 10% (vol/vol) FBS]; and HCC38 [RPMI with 10% (vol/vol) FBS] (3).

Flag-tagged USP36 (WT and the C131A mutant) plasmids were described (4, 5). V5-tagged USP36 (WT and the C131A mutant) plasmids were cloned by PCR into the pcDNA3-V5 vector. The primers were 5′-CGCGGATCCATGCCAATAGTGGATAAGTTGAAGG-3′ and 5′-CGCTCTAGATCAGCGGCGATAGCTGAGG-3′. All flag-tagged USP36 deletion mutants were constructed by inserting PCR products into the pcDNA3-2Flag vector. Flag-USP36^{res} expressing USP36 resistant to shRNA-3 was constructed by mutagenesis, in which the USP36 shRNA targeting sequence 5′-GCGGTCAGTCAGGATGCTATT-3′ was mutated to 5′-GCAGTGTCCCAAGACGCAATT-3′. pcDNA3–c-Myc was obtained from Addgene. All Flag-tagged and V5-tagged human c-Myc were constructed by subcloning c-Myc cDNA into pcDNA3-2Flag and pcDNA3-V5 vectors, respectively. The plasmids encoding Flag-tagged Fbw7α and Fbw7γ were generously provided by B. E. Clurman as described (6). For generation of a tet-inducible expression system, the Flag-USP36 and its C131A mutant were subcloned into the pcDNA4-TO (Invitrogen) vector to generate pcDNA4-TO–Flag-USP36 (WT and C131A) plasmids. His-tagged c-Myc (His–c-Myc) and His-USP36 (WT and C131A) bacterial expression vectors were constructed by PCR into the pPROEX-HT vector (Invitrogen). GST-USP36 (WT and C131A) bacterial expression vectors were constructed by PCR into the pGEX.4T.1 vector (Pharmacia).

Anti-Flag (M2, Sigma), anti-V5 (Invitrogen), anti–c-Myc (Y69 from abcam; N262 from Santa Cruz) antibodies were purchased. Other reagents, including MG132 (Peptide In.) and CHX (Calbiochem), were purchased.

Establishment of c-Myc– and USP36-Inducible and Stable Expression Cell Lines. The 293-TO–HA–c-Myc cell line was as described (7). To generate tet-inducible expression of c-Myc in U2OS cells, T-Rex–U2OS cells (Invitrogen) were transfected with pcDNA4-TO–c-Myc. The cells were then split into selection medium containing 50 μg/mL of hygromycin and 100 μg/mL of Zeocin, and selection was continued for 2 wk. Single colonies were isolated, expanded, and screened by immunoblot (IB) analysis for Dox-induced expression using anti-Flag antibodies. Similar procedures were used to establish tet-inducible expression of USP36 (WT and C131A) in HeLa-TO cells that have been stably transfected with pcDNA6-TR plasmid (Invitrogen). The cells were selected in selection medium containing 10 μg/mL of Blasticidin (Invitrogen) and 100 μg/mL of Zeocin. All of the cells were cultured in DMEM supplemented with 10% tetracycline system-approved FBS (Clontech) in the presence of proper selective antibiotics. Dox (2 μg/mL, Sigma) was used to induce tet-controlled gene expression. HeLa cell lines with stably transfected Flag–c-Myc^{WT} or Flag–c-Myc^{T58A} were generated by transfection with the corresponding plasmid followed by selection in culture medium containing 750 μg/mL G418. Single colonies were isolated, expanded, and screened for the protein expression by IB using anti-Flag antibodies.

Lentiviruses. To generate lentiviral expression of USP36, Flag-USP36 cDNA (WT and the C131A mutant) with an in-frame stop codon was amplified by PCR and inserted into the pENTR/D-TOPO vector using pENTR Directional TOPO Cloning Kits (Invitrogen). The cDNA was then cloned into pLenti4/V5-DEST using Gateway LR recombination reaction following the manufacturer's protocol (invitrogen). The resulting vector pLenti4/Flag-USP36 was then transfected with VSVG, pLP1, and pLP2 plasmids into 293FT cells using Calcium Chloride (Promega). The viruses were then used to infect cells in the presence of polybrene (6 μg/mL). The cells were harvested at 48 h posttransduction for IB analysis. Lentiviral vectors encoding shRNA against USP36 were purchased (Open Biosystems). The shRNA sequences are 5′-CGTCCGTATATGTCCCAGAAT-3′ (shRNA-1), 5′-GGAAGAGTCTCCAAGGAAA-3′ (shRNA-2), and 5′-GCGGTCAGTCAGGATGCTATT-3′ (shRNA-3, used for all experiments, except where indicated).

Transfection, IB, and Co-IP Analyses. Cells were transfected with plasmids using TransIT-LT1 reagents following the manufacturer's protocol (Mirus Bio Corporation). Cells were harvested at 36–48 h posttransfection and lysed in lysis buffer consisting of 50 mM Tris·HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 μg/mL pepstatin A, and 1 mM leupeptin. Equal amounts of clear cell lysate were used for IB analysis. Co-IP was conducted as described previously (1). Bound proteins were detected by IB using antibodies as indicated in figure legends.

RNA Interference. The 21-nucleotide siRNA duplexes with a 3′ dTdT overhang were synthesized by Dharmacon Inc. The target sequences (8) are 5′-CTACTCTAAACCATGGCTT-3′ (for Fbw7γ) and 5′-ACAGGACAGTGTTTACAAA-3′ (pan-Fbw7 siRNA for targeting all Fbw7 isoforms). These siRNA duplexes (100 nM) were introduced into cells using Lopofectamine2000 (Invitrogen) following the manufacturer's protocol.

RT-qPCR Analysis. Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen). Reverse transcriptions were performed as described (1). Quantitative real-time PCR was performed on an ABI StepOne real-time PCR system (Applied Biosystems) using SYBR Green Mix (Bio-Rad) as described previously (1). All reactions were carried out in triplicate. Relative gene expression was calculated using the $\Delta C\tau$ method following the manufacturer's instruction. The primers for *c-myc*, *prerRNA*, *5S RNA*, *E2F2*, and *GAPDH* were described (9, 10). Primers for USP36 are 5′-AGCACTTTTCCCCCAGAACTG-3′ and 5′-GGCTCCCAGATCTGCTGCTA-3′. cDNA from breast and lung cancer patient samples and patient-matched normal adjacent tissues used in Fig. 6*C* and Fig. S6*B* were provided by one of the authors (X.H.) with institutional review board approval.

GST-Fusion Protein Association Assays. His-tagged c-Myc protein was purified from bacteria through a Ni^{2+}-NTA (Qiagen) column. GST-fusion protein–protein association assays were conducted as described (1, 11). Briefly, purified His–c-Myc proteins (200 ng) were incubated with the glutathione-Sepharose 4B beads (Sigma) containing 200 ng of GST-USP36/1–800 and GST

alone, respectively. After washing, bound proteins were analyzed using IB with anti-Myc and anti-GST antibodies.

Cell Proliferation Assay. For visual examination of cell proliferation rates, equal amounts of cells infected with scrambled or one of the three USP36 shRNA-encoding lentiviruses were plated in DMEM containing 10% (vol/vol) FBS. Medium was changed every 3–5 d. After cells were cultured for 2–3 wk, the colonies were visualized by staining with 0.5% crystal violet in 50% (vol/vol) ethanol. Cell proliferation assay was also carried out on IncuCyte System (Essen Bioscience), which allows us to do kinetic, non-invasive imaging of cells in culture right inside the well-controlled incubator environment (11). The above lentiviral-infected cells were split into 12-well plates and monitored in IncuCyte System. Cell growth curve was calculated and presented as percentage of confluence from every 2-h phase-contrast imaging.

Flow Cytometry. Cells were fixed in ethanol and stained in 500 μL of PI (Sigma) stain buffer (50 μg/mL PI, 200 μg/mL RNase A, and 0.1% Triton X-100) at 37 °C for 30 min. The cells were analyzed for DNA content using a Becton Dickinson FACScan flow cytometer. Data were collected using the CellQuest program.

Soft Agar Assay. The bottom and top agar layers were 0.7% and 0.35% Nobel agar, respectively. For each cell line, 2×10^4 cells were plated in triplicate in a six-well plate. Culture medium on top of the agar was changed every 3–4 d. At 4 wk after plating, colonies were fixed and stained with 0.005% crystal violet in 50% (vol/vol) methanol/50% (vol/vol) PBS solution. Colonies were counted in 10 random microscopic fields using the EVOS FL cell imaging system (Advanced Microscopy Group).

Cell Fractionation. Cell fractionation assays were performed essentially as described (2). Briefly, cells were resuspended in a hypotonic buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT in the presence of protease inhibitors). Cell membranes were then broken using a dounce homogenizer (tight pestle) by 10 up and down strokes. After centrifugation, the supernatant was collected as the cytoplasmic fraction. The nuclear pellets were washed with buffer A and then resuspended in buffer S1, containing 0.25 M sucrose and 10 mM $MgCl_2$; layered over buffer S2, containing 0.35 M sucrose and 0.5 mM $MgCl_2$; and centrifuged at 1,430 × *g* for 10 min at 4 °C. The pelleted nuclei were resuspended in buffer S2, followed by sonication. The sonicated nuclei were then layered over buffer S3, containing 0.88 M sucrose and 0.5 mM $MgCl_2$, and centrifuged at 3,000 × *g* for 10 min at 4 °C. The pellet contained purified nucleoli and the supernatant represented the nucleoplasm (2). The nucleoli were then lysed in high salt RIPA buffer containing 50 mM Tris pH 7.5, 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and proteasome inhibitors in the presence of 80 U/mL DNase I on ice for 15–30 min. The lysates were then added with 2× the volume of RIPA buffer without salt, left on ice for an additional 10 min, followed by centrifugation at maximal speed for 15 min. The supernatant was collected as soluble nucleolar fraction for IP analysis (12).

Immunofluorescence Staining. Cells transfected with plasmids were fixed and stained with monoclonal anti-Flag antibody and polyclonal anti-NS antibodies followed by staining with Alexa Fluor 488 (green) goat anti-mouse antibody and Alexa Fluor 546 (red) goat anti-rabbit antibody (Invitrogen) as well as DAPI for DNA staining. Stained cells were analyzed under a Leica inverted fluorescence microscope.

In Vivo Ubiquitination Assay. In vivo ubiquitination assay under denaturing conditions was conducted using a Ni^{2+}-NTA PD method as previously described (1, 13). Briefly, cells transfected with indicated plasmids were treated with 40 μM MG132 for 6 h before harvesting. The cells were harvested at 48 h after transfection, and 20% of the cells were used for direct IB and the rest of cells were used for ubiquitination assays under denaturing conditions using Ni^{2+}-NTA PD. The bead-bound proteins were analyzed using IB.

In Vitro Deubiquitination Assay. Recombinant His-USP36 and its C131A mutant proteins were expressed in *Escherichia coli* and purified using the Ni^{2+}-NTA purification method. Ubiquitinated c-Myc was generated from 293 cells transfected with Flag–c-Myc and His-Ub using anti-Flag affinity purification (14). The ubiquitinated c-Myc was then incubated with 0.5 μM (final concentration) of purified His-USP36 (WT or the C131A mutant) in deubiquitination buffer consisting of 50 mM Tris-HCl (pH 8.0) and 10 mM DTT at 37 °C for 8 h. The reactions were resolved in SDS/PAGE gel followed by IB.

ChIP–qPCR. ChIP analysis was performed essentially as described (9) using anti–c-Myc (N262) antibodies. Immunoprecipitated DNA fragments were analyzed for USP36 promoter occupancy by qPCR amplification. The primers used were 5′-AATCTAGCCCCGCCTCTTAAA-3′ and 5′-TGTCACCGCGACGCTTAAA-3′ (A-exon 1), 5′- GAGCGGGAATGAGCCTAAATG-3′ and 5′-GCACAGGCCTCATCCACAT-3′ (B-intron 1), 5′-TAGCACCTGCCCAACCAAAC-3′ and 5′-CTACTGCGGCATGGAACCA-3′ (C-exon 2), and 5′-GAATTTGCCTCTGGGTACTCAAAC-3′ and 5′-CAGGAAGCAAGGGCAATCC-3′ (D-intron 6).

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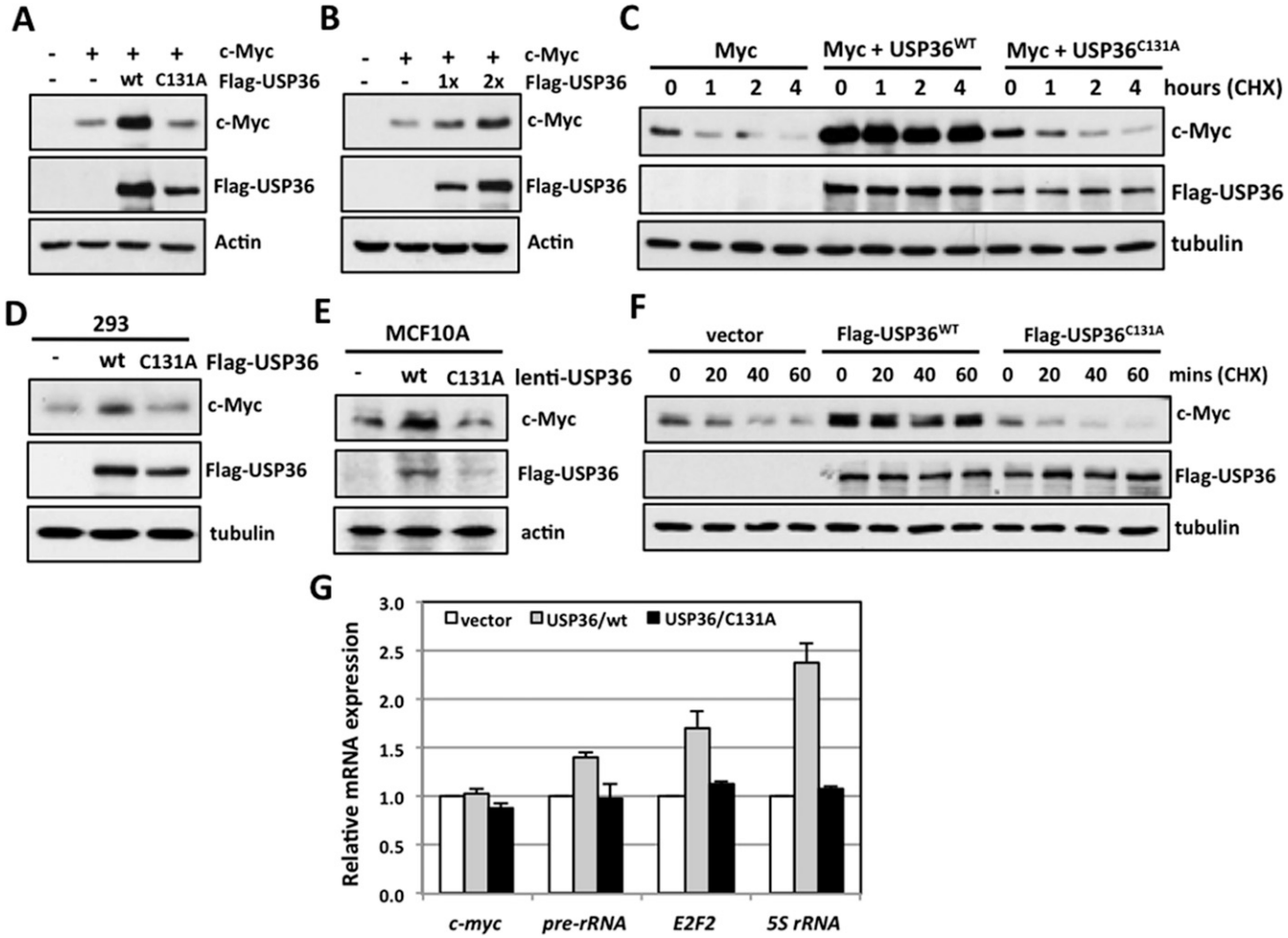

Fig. S1. USP36 stabilizes and activates c-Myc. (*A* and *B*) Overexpression of WT USP36, but not the C131A mutant, induces ectopically expressed c-Myc protein. HeLa cells transfected with c-Myc together with the control vector or different amounts of Flag-USP36 (*B*) or with control, WT, or C131A mutant Flag-USP36 (*A*) were assayed for the expression of indicated proteins by IB. (*C*) USP36 stabilizes ectopically expressed c-Myc. HeLa cells transfected with c-Myc together with control or Flag-USP36 (WT or the C131A mutant) for 48 h and then treated with 50 μg/mL CHX. The cells were harvested at different time points and assayed for the levels of c-Myc and tubulin by IB. (*D* and *E*) Overexpression of WT USP36, but not the C131A mutant, induces endogenous c-Myc protein. HEK293 (*D*) or MCF10A (*E*) cells transfected with control or Flag-USP36 (WT or the C131A mutant) were assayed for the expression of indicated proteins by IB. (*F*) USP36 stabilizes endogenous c-Myc. HEK293 cells were transfected with control, Flag-USP36^{WT} (1 μg), or Flag-USP36^{C131A} (3 μg) in 60-mm plates for 48 h and then treated with 50 μg/mL CHX. The cells were harvested at different time points and assayed for the expression of indicated proteins by IB. (*G*) USP36 stimulates c-Myc activity. The 293 cells transfected with control or Flag-USP36 (WT or the C131A mutant) plasmids were assayed for the expression of indicated Myc target genes by RT-qPCR analysis.

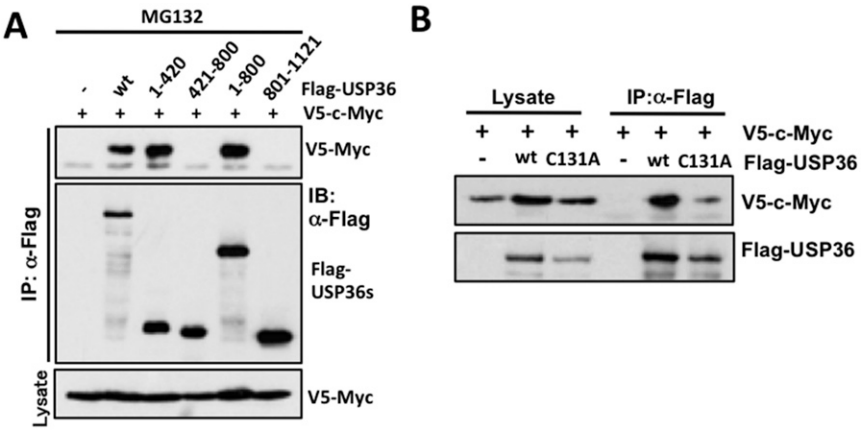

Fig. S2. Characterization of the USP36–c-Myc interaction. (*A*) c-Myc binds to the N-terminal USP domain of USP36. HEK293 cells were transfected with V5–c-Myc together with control or Flag-USP36 (WT or its deletion mutants). The cells were treated with MG132 (40 μM) for 6 h before harvesting. The cell lysates were immunoprecipitated with anti-Flag antibody followed by IB with anti-Flag and anti-V5 antibodies. (*B*) The catalytic mutant USP36^{C131A} binds to c-Myc. HEK293 cells transfected with V5–c-Myc together with control, WT USP36, or USP36^{C131A}, were subjected to IP with anti-Flag followed by IB using anti-Flag or anti-V5 antibodies.

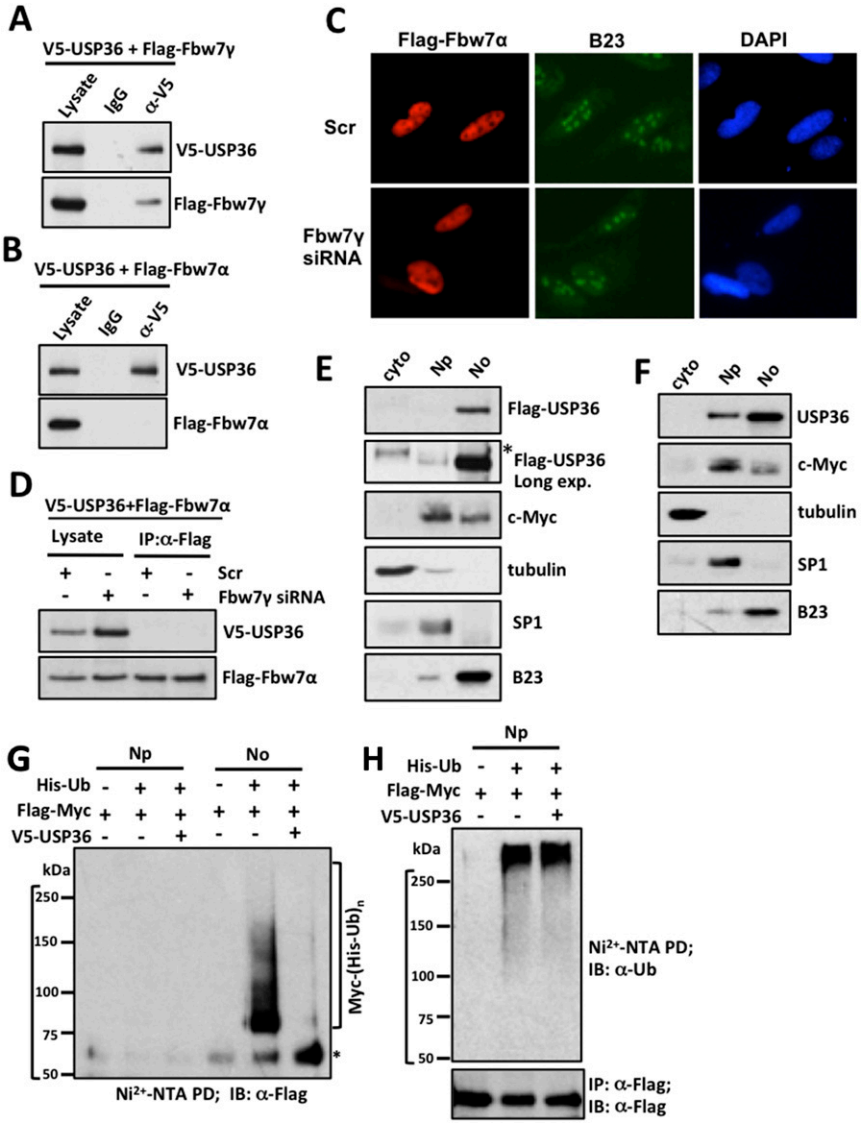

Fig. S3. USP36 is localized in the nucleolus and interacts with the nucleolar Fbw7γ, but not the nucleoplasmic Fbw7α. (*A* and *B*) HeLa cells transfected with V5-USP36 together with Flag-Fbw7γ (*A*) or Flag-Fbw7α (*B*) were subjected to co-IP with anti-V5 or control IgG, followed by IB. (*C* and *D*) Knockdown of Fbw7γ does not result in the relocalization of Fbw7α from the nucleoplasm into the nucleolus and its binding to USP36. HeLa cells were transfected with Flag-Fbw7α together with scrambled or Fbw7γ siRNA. The cells were subjected to IF staining using anti-Flag (Fbw7α) and anti-B23 (*C*) and co-IP using anti-Flag followed by IB (*D*). (*E* and *F*) USP36 is predominantly expressed in the nucleolus. HeLa cells overexpressing Flag-USP36 (*E*) or parental HeLa cells (*F*) were subjected to cell fractionation to isolate the cytoplasm (cyto), nucleoplasm (Np), and nucleolar (No) fractions, followed by IB. (*G* and *H*) USP36 regulates c-Myc ubiquitination in the nucleolus, but not the nucleoplasm. HeLa cells transfected with indicated plasmids were treated with 40 μM MG132 for 6 h before harvesting, followed by cell fractionation. The isolated nucleoplasmic fraction and the nucleoli were subjected to in vivo ubiquitination assays using Ni^{2+}-NTA PD, followed by IB using anti-Flag antibody (Fig. S3*G*). Asterisks indicate nonspecific PD of native c-Myc. The levels of ubiquitinated c-Myc are undetectable in the nucleoplasmic fraction, although the total ubiquitinated proteins were efficiently pulled down by the Ni^{2+}-NTA purification as determined by IB using anti-Ub antibodies (Fig. S3*H*). The levels of c-Myc in the nucleoplasmic fraction were also shown by IP with anti-Flag followed by IB.

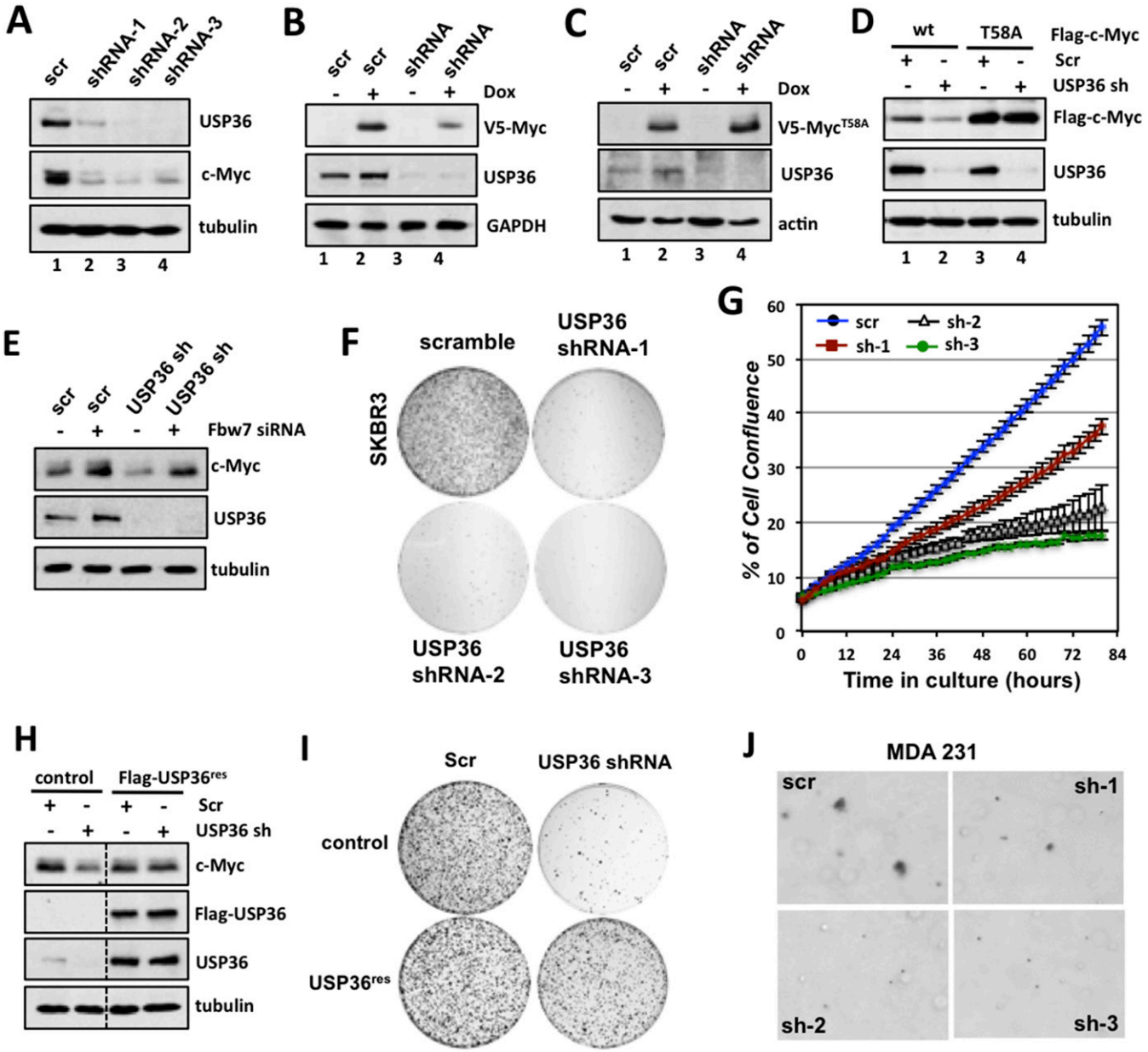

Fig. S4. Knockdown of USP36 reduces c-Myc levels and suppresses cell proliferation. (*A*) Knockdown of USP36 reduces the levels of endogenous c-Myc. MDA-MB-231 cells infected with control or USP36 shRNA-encoding lentiviruses, followed by IB detection of the indicated proteins. (*B–D*) Knockdown of USP36 reduces the levels of WT, but not the T58A mutant, c-Myc. MCF10A-TO–Myc^{WT} (*B*) or MCF10A-TO–Myc^{T58A} (*C*) cells were infected with scrambled or USP36 shRNA for 72 h. The cells were then cultured in the absence or presence of dox for 12 h and assayed for the expression of c-Myc and USP36 by IB. HeLa cells stably expressing WT c-Myc or c-Myc^{T58A} were infected with scrambled or USP36 shRNA lentiviruses, followed by IB (*D*). (*E*) Knockdown of Fbw7 abolishes the c-Myc reduction mediated by USP36 knockdown. HeLa cells were transfected by scrambled or pan-Fbw7 siRNA, followed by infection with scrambled or USP36 shRNA lentiviruses. The cells were then assayed for the expression of indicated proteins by IB. (*F* and *G*) Knockdown of USP36 suppresses cell proliferation. SKBR3 cells were infected with control or USP36 shRNA-encoding lentiviruses. The cells were cultured for up to 3 wk. The colonies were visualized by staining with crystal violet (*F*). The infected HeLa cells were also cultured in medium, and cell confluence was measured over time using IncuCyte System (*G*). (*H* and *I*) Overexpression of shRNA-resistant Flag-USP36^{res} rescues the reduction of c-Myc levels and the cell growth inhibition by USP36 knockdown. HeLa cells expressing Flag-USP36^{res} or control vector were infected with scrambled or USP36 shRNA-encoding lentiviruses. The cells were assayed for the protein expression by IB (*H*) and cell proliferation by colony formation (*I*). (*J*) Soft agar colony formation assays. MDA231 breast cancer cell lines were infected with control or USP36 shRNA-encoding lentiviruses, followed by colony formation assay in soft agar. The representative images are shown.

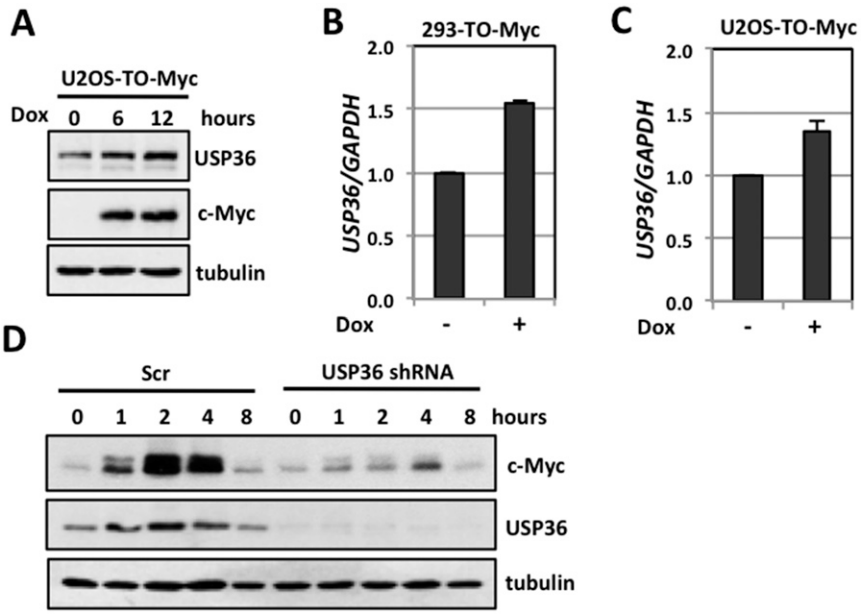

Fig. S5. USP36 is a target gene of c-Myc. (*A*) c-Myc induces the expression of USP36 protein in U2OS-TO–Myc cells. The cells were cultured in the presence of dox for different times as indicated, followed by IB. (*B* and *C*) c-Myc induces the expression of USP36 mRNA. The 293-TO–Myc (*B*) or U2OS-TO–Myc (*C*) cells were cultured in the presence of dox for 4 h, followed by detection of USP36 mRNA by RT-qPCR. (*D*) Knockdown of USP36 abolishes the c-Myc induction following serum stimulation. HeLa cells infected with control or USP36 shRNA-encoding lentiviruses were cultured in 0.2% FBS containing medium for 48 h and then stimulated with 20% FBS. The cells were harvested at the indicated time points and assayed for expression of c-Myc and USP36 by IB.

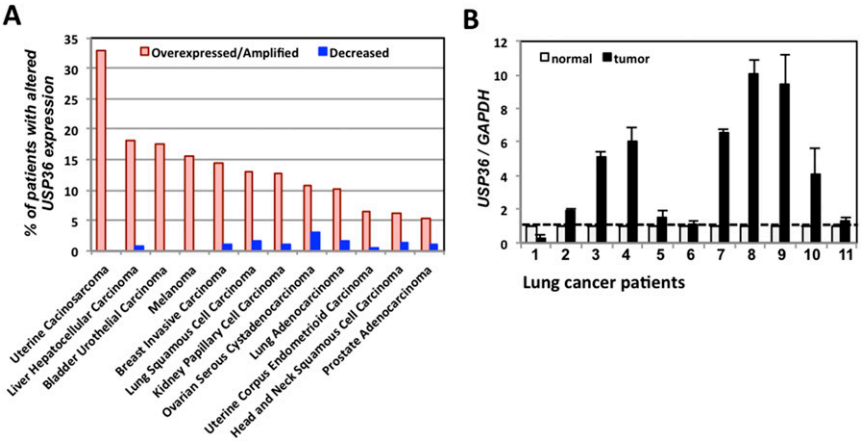

Fig. S6. USP36 is overexpressed in human cancers. (*A*) USP36 expression is altered in various human cancers. Data are obtained from cBioPortal, showing the percentage of USP36-overexpressed or -decreased cases in indicated cancers. (*B*) USP36 is overexpressed in a subset of primary human lung cancers. Cancer tissues from lung cancer patients as well as patient-matched normal adjacent tissues were assays for USP36 mRNA expression by RT-qPCR.