Supporting Information

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SI Text

SI Materials and Methods. ***Protein expression in bacteria and purification of GST and V5-tagged proteins.*** An overnight culture of bacteria transformed with the respective plasmid was diluted 1:10 in fresh Luria–Bertani medium supplemented with 100 μg/ml ampicillin. Cells were grown to midlog phase and protein expression was induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM and bacteria were grown for an additional 5–7 h. Cells were pelleted by centrifugation, resuspended in 1/20 volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and lysed by mild sonication. Triton-X-100 was added to a final concentration of 1% and the proteins were solubilised by gently rotation of the mixture at room temperature for 30 minutes. The extract was cleared by centrifugation at 10000 × *g* for 5 min at 4 °C. V5-tagged proteins were not further purified. GST-tagged proteins were coupled for 4 h to Glutathione sepharose at 4 °C. The Glutathione-sepharose-GST-protein mixture was pelleted by centrifugation at 1000 rpm for 5 min at 4 °C, washed 5 times with PBS and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0). Expression of proteins in insect cells was performed according to standard procedures. Flag-tagged Mdm2 was purified using Anti-Flag® M2 agarose affinity gel (Sigma) according to the manufacturers recommendations.

In vitro Mdm2/proteasome interaction assay. Flag- mdm2 was expressed in SF9 cells as described (20). Cells were washed twice with cold phosphate-buffered saline (PBS), and harvested in lysis buffer (20 mM Hepes pH 7.4, 0.5% Triton X-100, 2 mM $MgCl_2$, 10 μM $ZnCl_2$, 2 mM NEM, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM NaF, 2 mM sodium orthovanadate, 20 μM MG132), containing 240 mM NaCl. Cell lysates were kept on ice for 30 min and centrifuged at 4 °C. Supernatants were transferred to fresh microtubes and protein concentrations were determined by Bradford assay (BioRad). Prewashed 20 μL anti-flag M2 agarose beads (Sigma) were incubated with 500 ug of the lysates on a rotating wheel at 4 °C for 4 h. Lysates were washed three times in hypotonic buffer (10 mM Hepes, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.2 μg/uL BSA). Where indicated, the samples were further mock treated or incubated with 100 units of lambda protein phosphatase (New England Biolabs) alone or with 10 mM sodium orthovanadate (Sigma) and 50 mM sodium fluoride (Sigma) for 1 h at 65 °C in hypotonic buffer. Where indicated, mock or phosphatase-treated samples were washed 3x in kinase buffer (20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.5 mM DTT, 10 μM ATP) and then mock incubated or incubated with 4 ng GSK-3β kinase (Upstate Biotech) for 1 h at RT. The samples were then washed three times with NP-40 buffer and once with hypotonic buffer, and incubated overnight with 1 μL of 26S proteasome. The samples were washed five times with hypotonic buffer and eluted with Flag peptide (Sigma) for 30 minutes at room temperature. Eluted material was analyzed by SDS-PAGE and Western blot.

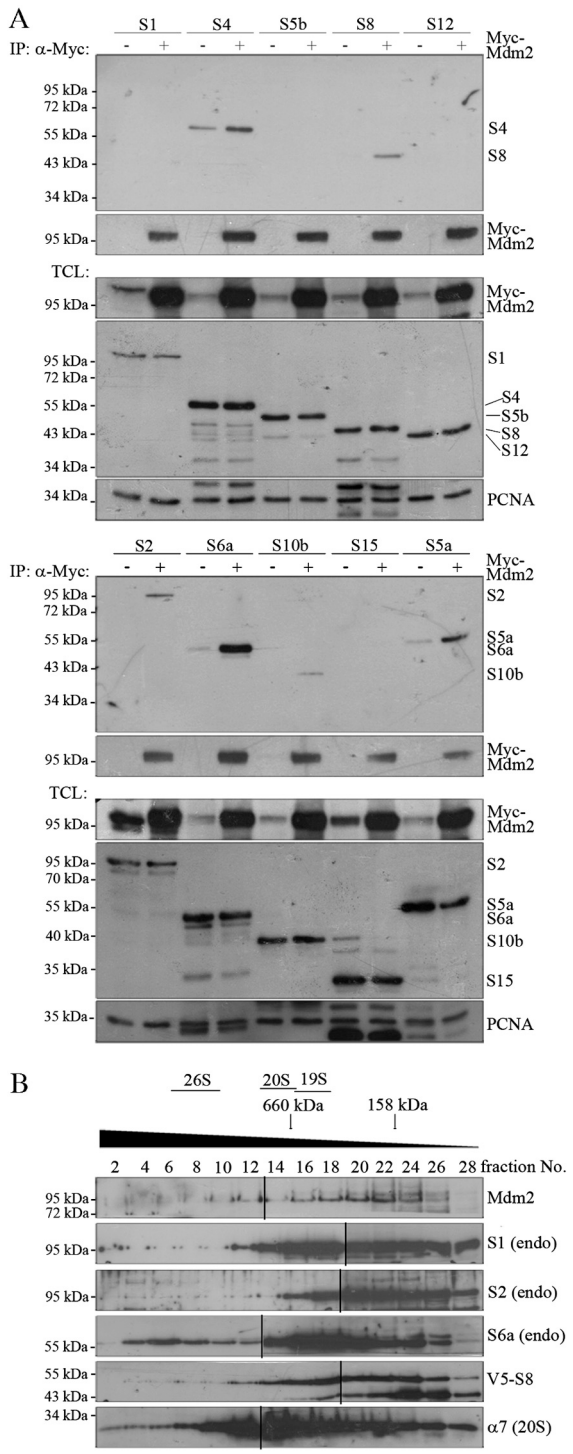

Fig. S1. Mdm2 associates with some but not with all proteasomal proteins. (*A*) H1299 cells were transfected with 6 μg of a plasmid encoding Myc-tagged Mdm2 and varying amounts of plasmids encoding the indicated V5-tagged proteins of the 19S complex (adjusted to similar expression levels). **IP:** α-**Myc**: Mdm2 was precipitated using the antiMyc antibody 9E10 and associated proteasomal proteins were detected by Western blotting using an anti-V5 antibody. After development of the blots, the membranes were rehybrised with the 9E10 antiMyc antibody. **TCL** (total cell lysate): 14 μg of cellular lysate were separated by SDS-PAGE and blotted. Expression of proteasomal proteins was detected by incubation with an anti-V5 antibody. After development of the blot, the membrane was divided into two parts. The upper part was hybridized with an antiMyc antibody, the lower one with an antibody recognizing proliferating cell nuclear antigen (PCNA) (*B*) H1299 cells were transfected with 7 μg of a plasmid encoding Mdm2 together with 3 μg of a plasmid encoding V5-tagged S8. Cell extracts were separated by sucrose gradient. 28 fractions were collected and 25 μL of every second fraction were loaded onto an SDS-PAGE gel. Mdm2, V5-tagged S8, endogenous (endo) S1, S2, S6a and the alpha7 subunit of the 20S-core were determined by Western blotting. The black lines indicate where two gels were spliced together.

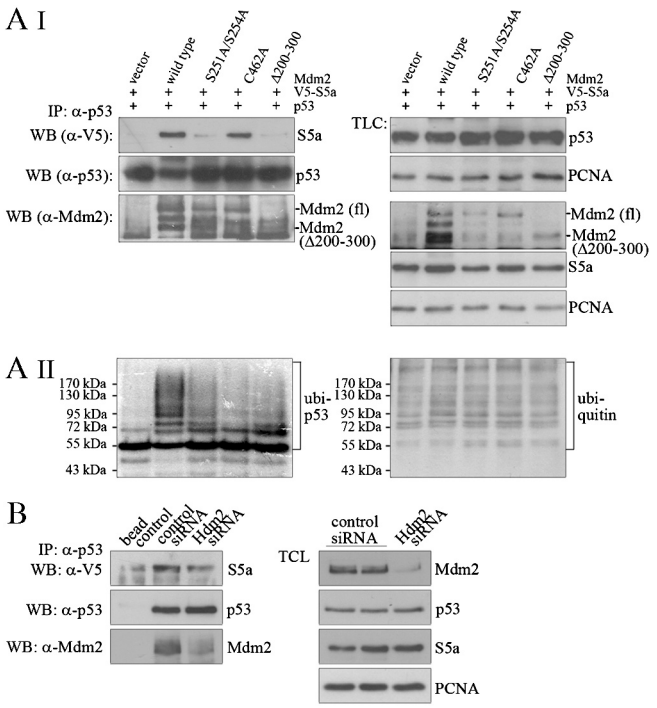

Fig. S2. The association of p53/Mdm2 with the proteasome is independent of ubiquitylation. (*A*) H1299 cells were transfected with 7 μg of a plasmid encoding p53 together with 7 μg of a plasmid encoding wild type Mdm2 or the indicated Mdm2 mutants, 1 μg of a plasmid encoding His-tagged ubiquitin and varying amounts of V5-tagged S5a to adjust for similar levels, or with vector DNA for control. 48 h after transfection, cells were harvested and the lysate was divided into two parts. **I. (IP)**: p53 was precipitated using the anti-p53 antibody Pab421 coupled to protein A agarose. The beads were washed and loaded onto a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred onto Immobilone blotting membrane and hybridized with an HRP-conjugated anti-V5 antibody to monitor coprecipitating proteasomes. Afterward, the blot was hybridized with an HRP-conjugated anti-p53 antibody to monitor IP-efficiency and afterward with an anti-Mdm2 antibody. **TCL**: 2×25 μg of cell lysate were separated by 10% SDS-PAGE gels and transferred onto Immobilone blotting membranes. One of the membranes was hybridized with an antibody directed against p53 and with an antibody directed against PCNA for loading control. The second membrane was first hybridized with an antibody directed against V5 and afterward with an antibody directed against Mdm2 and with an antibody directed against PCNA for loading control. fl: full length protein. **II**: Ubiquitylated proteins were purified by adsorption to Ni2+ agarose. The proteins were separated by two 10% SDS-PAGE gels and probed for the presence of p53 and for ubiquitinated proteins by standard Western blotting. (*B*) U2OS cells were transfected with an siRNA directed against Mdm2, or with a control siRNA. P53 was precipitated and associated proteasomes were determined by Western blotting with an antibody directed against S5a.

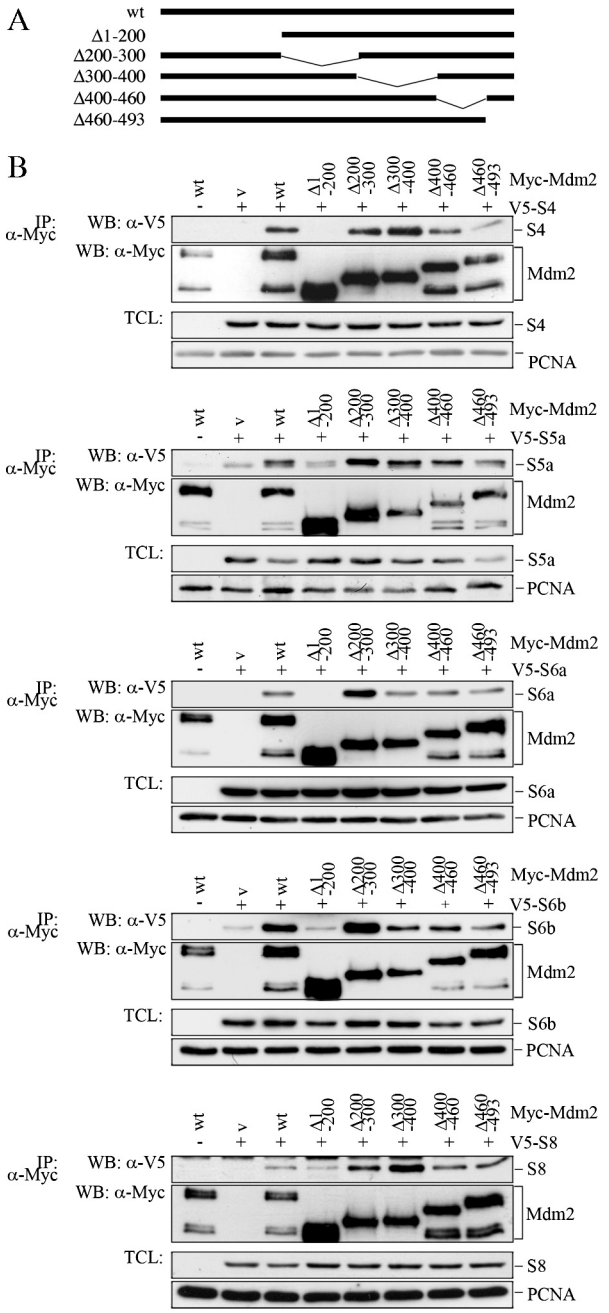

Fig. S3. Mapping of the 19S interaction site on the Mdm2 protein. (*A*) Schematic drawing of the employed Mdm2 deletion mutants. (*B*) 293 cells were transfected with 7.5 μg of a plasmid encoding Myc-tagged wild type or mutant Mdm2 harboring the indicated deletions together with 7.5 μg of a plasmid encoding V5-tagged S4 or S5a or S6a or S6b or S8, respectively. 24 h after transfection, cells were harvested. **IP: α-Myc**: Myc-Mdm2 was immunoprecipitated and associated proteasomal proteins were determined by Western blotting using an anti-V5 antibody. After development of the blots, the membranes were hybridized with the 9E10 antibody, targeted against Myc Mdm2. **TCL**: 50 μg of cellular lysate were separated by SDS-PAGE and blotted. Expression of proteasomal proteins was detected by incubation with an anti-V5 antibody. After development of the blot, the membrane was divided into two parts. The upper part was hybridized with an antiMyc antibody, the lower one with an antibody recognizing PCNA.

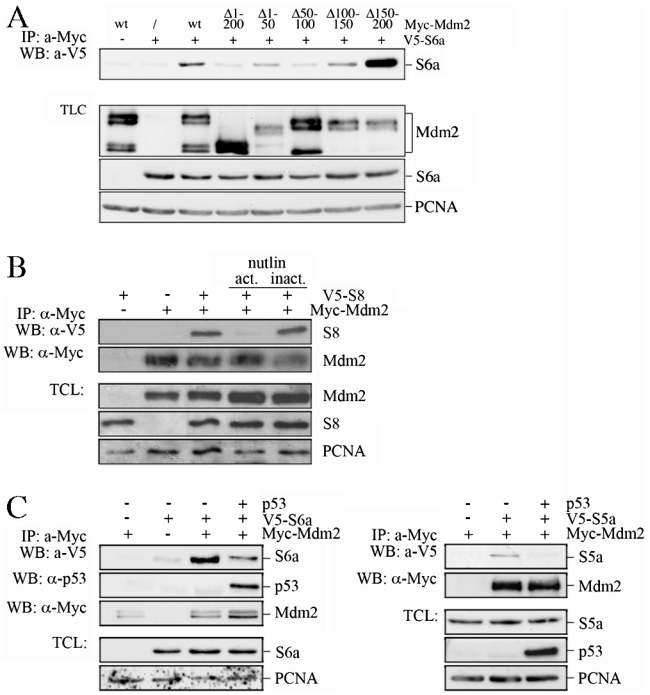

Fig. S4. Mapping of the 19S interaction site on the Mdm2 N-terminal domain. (*A*) 293 cells were transfected with 7.5 μg of a plasmid encoding Myc-tagged wt or mutant Mdm2 harboring the indicated deletions together with a plasmid encoding V5-tagged S6a. 24 h after transfection, cells were harvested. **IP: α-Myc**: Myc-Mdm2 was immunoprecipitated and associated S6a was determined by Western blotting. **TCL**: 50 μg of cellular lysate were separated by SDS-PAGE and blotted. Expression of S6a was determined by incubation with an anti-V5 antibody. After development of the blot, the membrane was divided into two parts. The upper part was hybridized with an anti-Myc antibody, the lower one with an antibody recognizing PCNA. (*B*) H1299 cells were transfected with 7.5 μg of a plasmid encoding Myc-tagged Mdm2 together with 7.5 μg of a plasmid encoding V5-tagged S8 or with vector DNA for control. 48 h after transfection, active nutlin-3 or the inactive enantiomer were added for 2 h. **IP: α-Myc**: Myc-Mdm2 was precipitated and associated S8 was determined by Western blotting. **TCL**: 5 μg of cellular lysate were separated by SDS-PAGE and blotted. Expression of S8 was determined by incubation with an anti-V5 antibody. After development of the blot, the membrane was divided into two parts. The upper part was hybridized with an anti-Myc antibody, the lower one with an antibody recognizing PCNA. (*C*) H1299 cells were transfected with 5 μg of a cDNA encoding V5-tagged S5a or S6a together with 5 μg of a plasmid encoding Myc-tagged Mdm2 and 5 μg of a plasmid encoding p53 or vector DNA for control. 24 h after transfection, cells were harvested. **IP: α-Myc**: Myc-Mdm2 was precipitated and associated proteasomal proteins were determined by Western blotting. **TCL**: 50 μg of cellular lysate were separated by SDS-PAGE and blotted. Expression of S5a or S6a, respectively, were determined by incubation with an anti-V5 antibody. After development of the blot, the membrane was divided into two parts. The upper part was hybridized with an anti-Myc antibody, the lower one with an antibody recognizing PCNA.

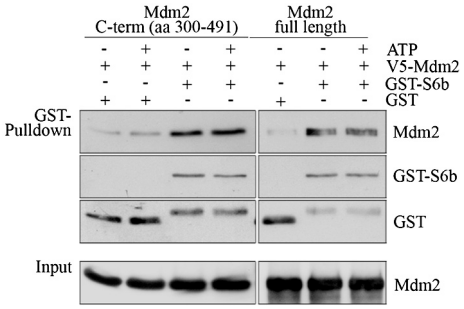

Fig. S5. Binding of Mdm2 to the proteasome is independent of ATP. Bacterially expressed V5-tagged full length human Mdm2 or the C-terminal domain (aa 300–493) were mixed with bacterially expressed and purified GST or S6b fused to GST in the presence and absence of 1 mM ATP. GST was pulled down and associated Mdm2 was detected by Western blotting.

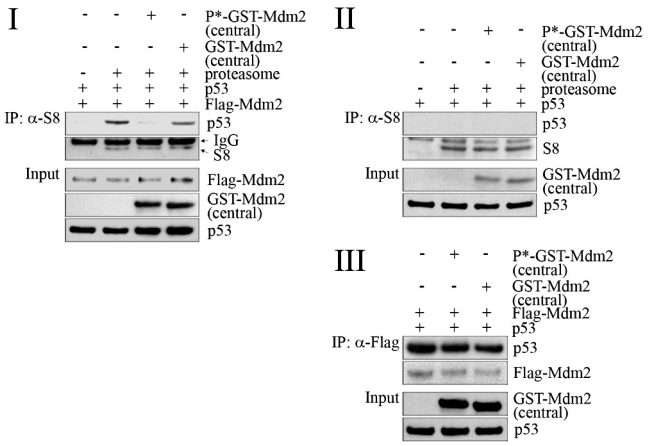

Fig. S6. The presence of phosphorylated central domain of Mdm2 interferes with p53/Mdm2/proteasome ternary complex formation **(I)** Flag-tagged Mdm2 (aa 1–491) and p53, expressed in insect cells, were mixed with bacterially expressed and purified GST or GST-fused to the central domain of Mdm2 (aa 200–299) and 26S proteasomes. Where indicated, the central domain was phosphorylated with CKIdelta prior to addition to the mixture. S8 was precipitated and associated p53 was detected by Western blotting. **(II)** p53, expressed in insect cells, was mixed with bacterially expressed and purified GST or GST-fused to the central domain of Mdm2 (aa 200–299) and 26S proteasomes. Where indicated, the central domain was phosphorylated with CKIdelta prior to addition to the mixture. S8 was precipitated and associated p53 was detected by Western blotting. **(III)** Flag-tagged Mdm2 (aa 1–491) and p53, expressed in insect cells, were mixed with bacterially expressed and purified GST or GST-fused to the central domain of Mdm2 (aa 200–299). Where indicated, the central domain was phosphorylated with CKIdelta prior to addition to the mixture. Mdm2 was precipitated and associated p53 was detected by Western blotting.

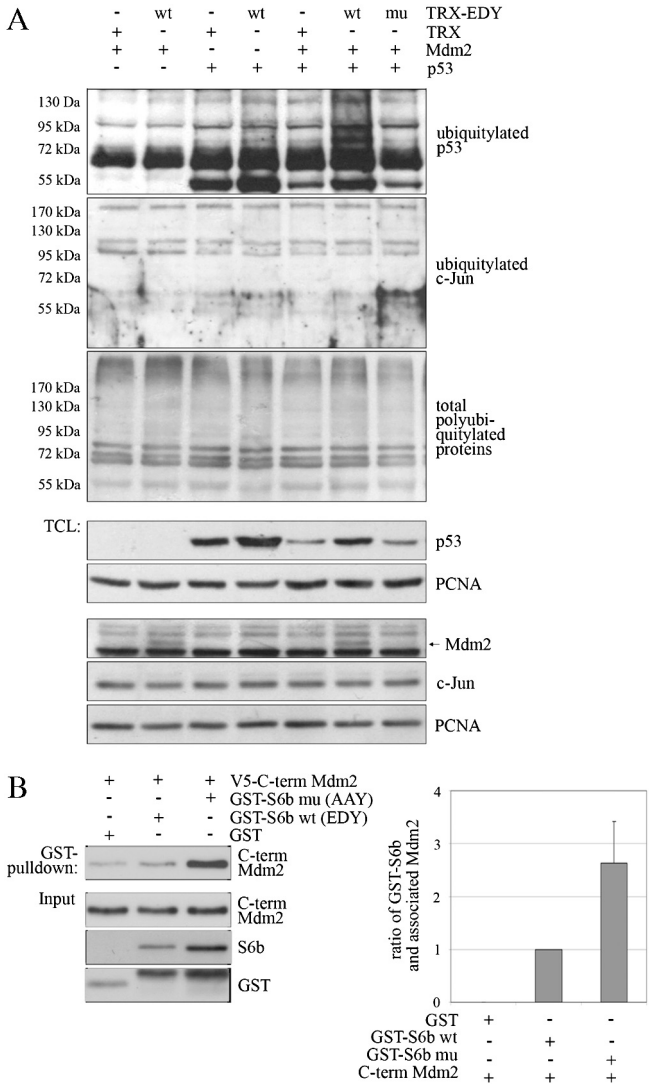

Fig. S7. Specificity control for an EDY-containing peptide. (*A*) H1299 were transfected with 1 μg of a plasmid encoding His-tagged ubiquitin, 0.4 μg of a plasmid encoding p53 and with 1.2 μg of a plasmid encoding Mdm2. Where indicated, cells were additionally transfected with 30 μg of a plasmid encoding thioredoxin, thioredoxin fused with an EDY-containing peptide of Mdm2 (aa 245–264, wt) or with thioredoxin fused with a peptide where the tyrosin of the EDY-motif was replaced with an alanine (mu). Ubiquitylated proteins were purified by adsorption to Ni-agarose and probed for the presence of p53, c-Jun or total ubiquitylated proteins. **TCL**: An aliquot of the cells was lysed and 40 μg of the lysate were probed for the presence of Mdm2 and c-Jun or for p53. PCNA was determined for loading control. (*B*) Bacterially expressed V5-tagged C-terminus of human Mdm2 (aa 300–491) was mixed with bacterially expressed and purified GST or GST-fused to a peptide of S6b (aa 353–368) where the EDY-motif was replaced with AAY. GST was pulled down with glutathione sepharose and associated Mdm2 was determined by Western blotting. The signals for S6b (input) and Mdm2 (pull down) of three independent experiments were quantified. The ratios were calculated and plotted. The ratio between wt S6b and pulled down Mdm2 was set to 1.

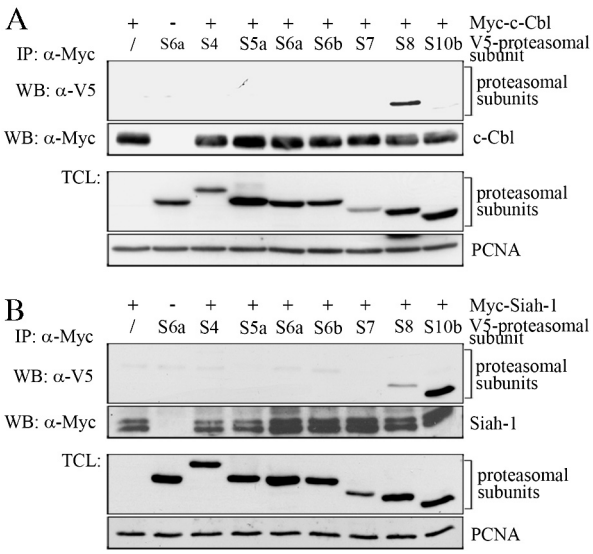

Fig. S8. The ubiquitin ligases c-Cbl and Siah-1 associate with the proteasome. 293 cells were transfected with 7.5 μg of a plasmid encoding Myc-tagged c-Cbl (*A*) or Siah-1 (*B*) and 7.5 μg of a plasmid encoding the indicated V5-tagged proteins of the 19S complex. Mdm2 was precipitated and associated proteasomal proteins were detected by Western blotting.