SUPPLEMENTAL FIGURE LEGENDS

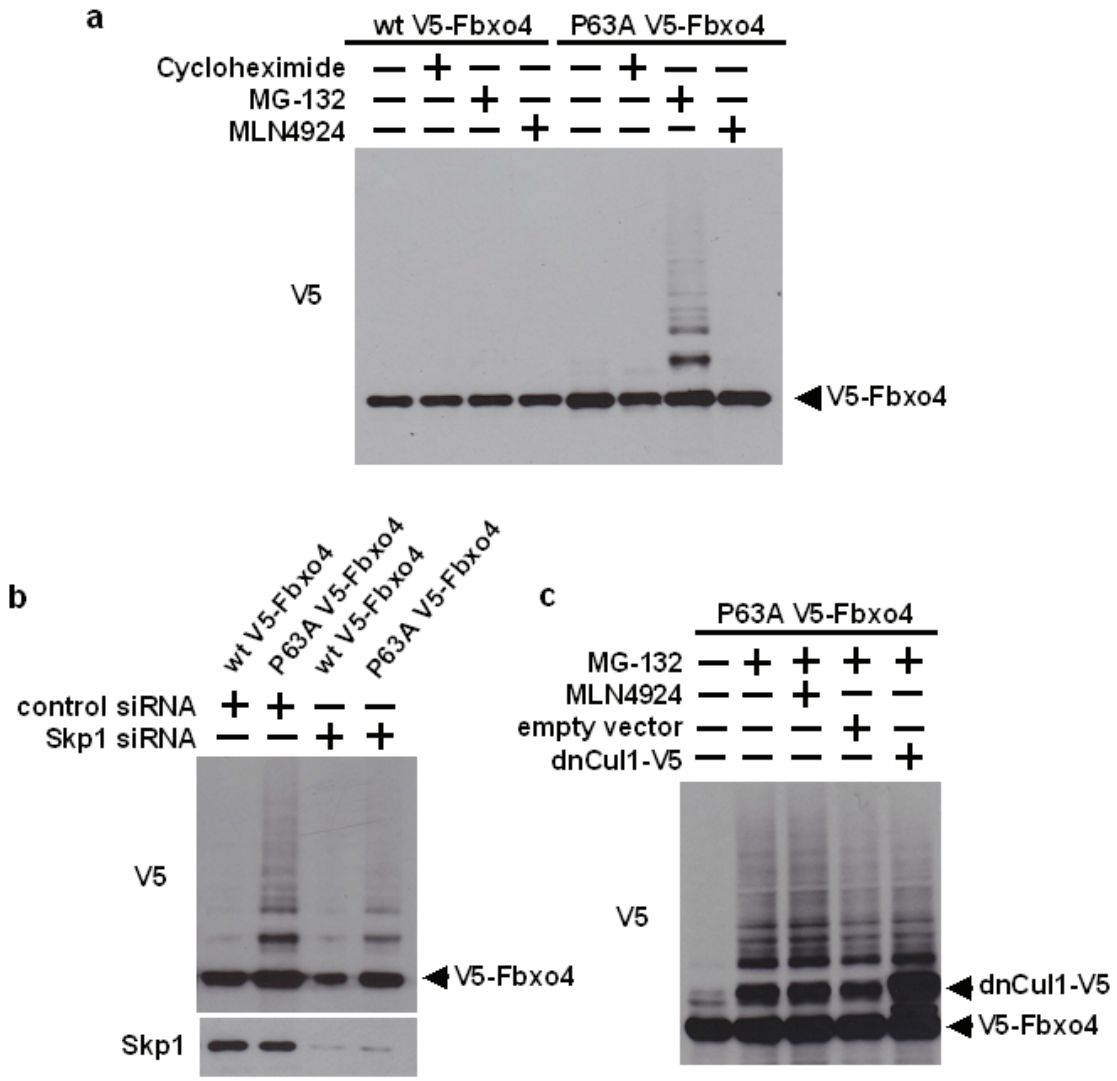

Fig.S1 P63A mutation induces Fbxo4 polyubiquitination. (a) HEK293T cells transfected with the wild type or P63A V5-Fbxo4 plasmids were treated for 6 hours with 40 μM cycloheximide, 20 μM MG-132 or 3 μM MLN4924 and cell lysates analyzed by Western blotting with V5 antibody. (b) Cells were transfected with negative control or Skp2 siRNA for three days and with wild type or mutant V5-Fbxo4 or V5-Fbxo4 for the last two days. MG-132 (20 μM) was added to all cells 6 hours before cell lysis and cells lysates analyzed by Western blotting with the indicated antibodies. (c) Cells were transfected with P63A V5-Fbxo4 and cotransfected with empty vector or dominant-negative Cul1 (dnCul1) expression plasmids and treated with MG-132 (20 μM) and MLN4924 (3 μM) for the last 6 hours before cell lysis as indicated.

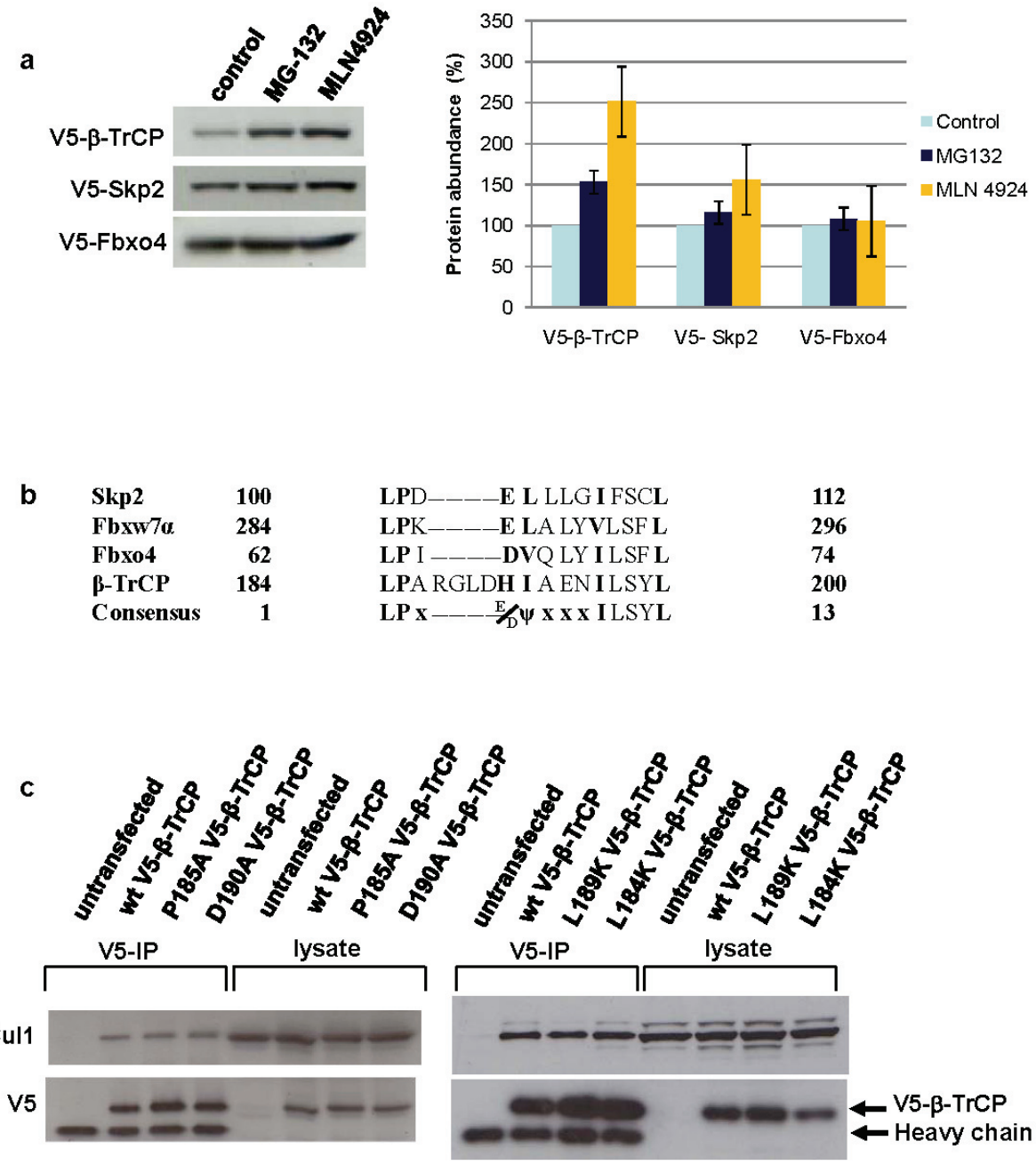

Fig.S2 Role of the conserved F-box proline residue in β-TrCP. (a) HEK293T cells were transfected with the indicated expression plasmids and treated for 6 hours with 20 μM MG-132, 3 μM MLN-4924, or DMSO (control). Cell lysates were analyzed by Western blotting with V5 antibody. The right panel shows the quantification of the V5-β-TrCP, V5-Skp2 and V5-Fbxo4 abundant by densitometry. The results represent the average of three independent experiments (N = 3 + SEM). (b) Amino acid sequence alignment of the N-terminal portion of the F-box domain of the indicated human F-box proteins. (c) Cells were transfected with the indicated plasmids. Cell lysates were subjected to V5 immunoprecipitation and immunoprecipitates analyzed by Western blotting with Cul1 and V5 antibodies.

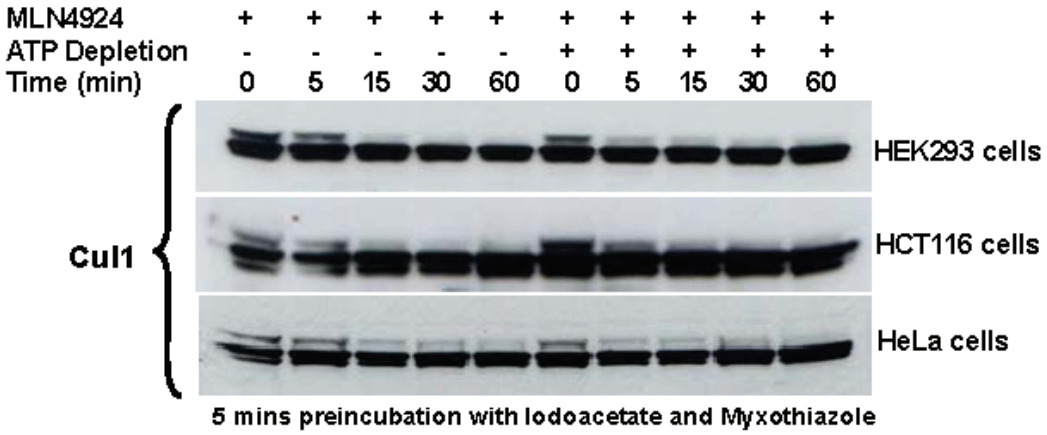

Fig.S3 *In vivo* cullin deneddylation rates in the presence and absence of ongoing substrate ubiquitination. HEK293, HCT116 and HeLa cells were pre-treated for 5 mins with myxothiazol (1 μM) and iodoacetate (2.5 mM) prior to addition of MLN4924 at time zero. The cells were lysed at the indicated time points and cell lysates analyzed by Western blotting with Cul1 antibody.

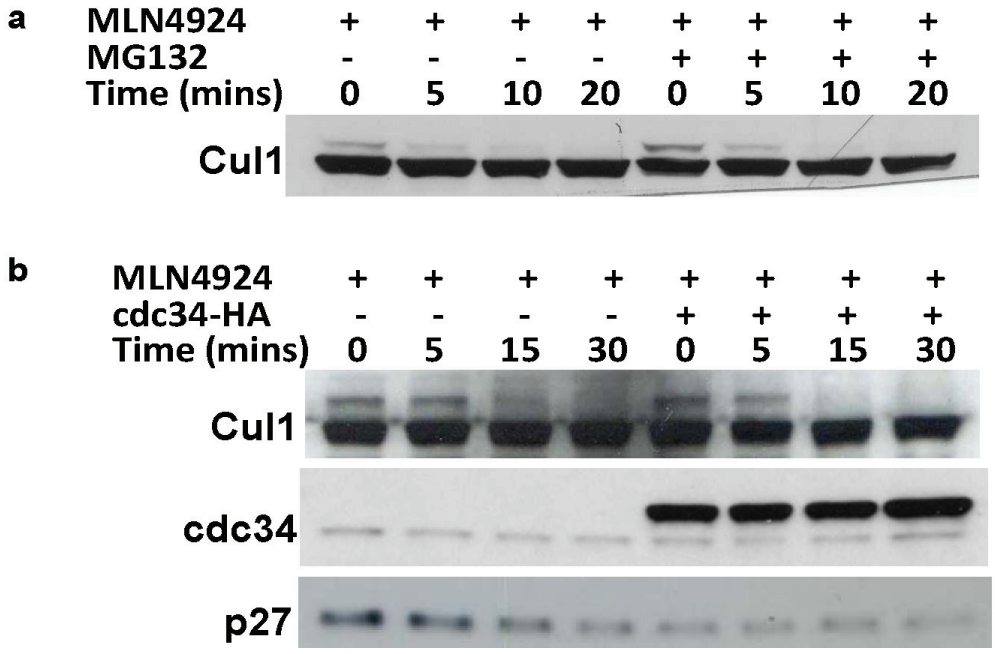

Fig.S4 In vivo Cul1 deneddylation. Cullin deneddylation rates were determined by growing HEK293 cells in 12-well plates. 3 μM MLN4924 was added at time zero to cells that were pretreated with 20 μM MG-132 for 20 min (a), transfected with empty vector or cdc34-HA expression plasmid (b), as indicated. Cells were lysed at the indicated time-points and cell lysates were analyzed by Western blotting with the indicated antibodies.

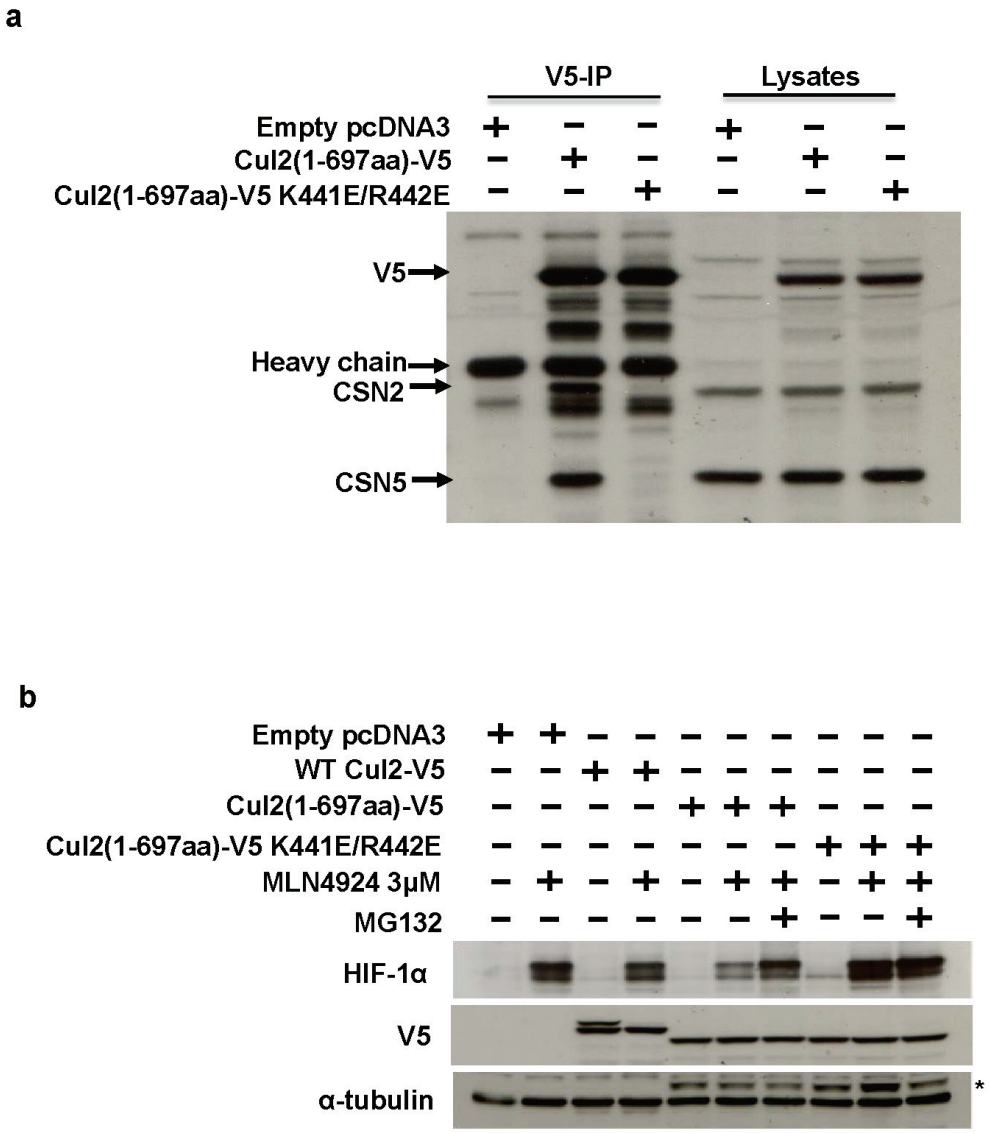

Fig.S5 Effect of K441E/R442E mutation in the Cul2 extreme C-terminal deletion mutant. (a) HEK293T cells were transfected in 60-mm cell culture plates for 2 days with expression constructs for the extreme C-terminal deletion mutants of Cul2, as indicated at the top of each panel. The KR mutant of Cul2 corresponds to the K441E/R442E mutant of Cul2 which is unable to bind to the CSN complex. The lysates were subjected to V5 immunoprecipitation (IP), Immunoprecipitates and aliquots of the cell lysates were analyzed by Western blotting with the indicated antibodies. (b) HEK293T cells were transfected with WT Cul2-V5, Cul2(1–697 aa)-V5, Cul2(1-697aa)-V5 K441E/R442E and empty vector for 24 h. Prior to lysis, cells were treated with dimethyl sulfoxide (DMSO) as vehicle control or with 1 μM of MLN4924 for 4 h to inhibit the intact cellular neddylation pathway, where indicated. Equal amounts of cell lysates were then prepared and subjected to Western blot analysis with the indicated antibodies. An asterisk (*) indicates a nonspecific band associated with anti-α-tubulin.