Supplementary Materials:

A

LHerhaus 120510 H 14 GFP-OTUB1 sample

m/z	Mass	Mascot Score	Retention Time	MS2 Scan Number
992.7518	2975.2314	87	22.06	1840
K.QEPLGSDSEGVNCLAYDEAIMAQQDR.I + P (ST)				

LHerhaus 120510 H 23 GFP-OTUB1 plus TGFbeta sample

m/z	Mass	Mascot Score	Retention Time	MS2 Scan Number
992.7512	2975.2314	71	22.16	1863
K.QEPLGSDSEGVNCLAYDEAIMAQQDR.I + P (ST)				
1488.6161	2975.2314	35	22.18	1867
K.QEPLGSDSEGVNCLAYDEAIMAQQDR.I + P (ST)				
998.0828	2991.2263	56	20.70	1657
K.QEPLGSDSEGVNCLAYDEAIMAQQDR.I + Ox (M); P (ST)				

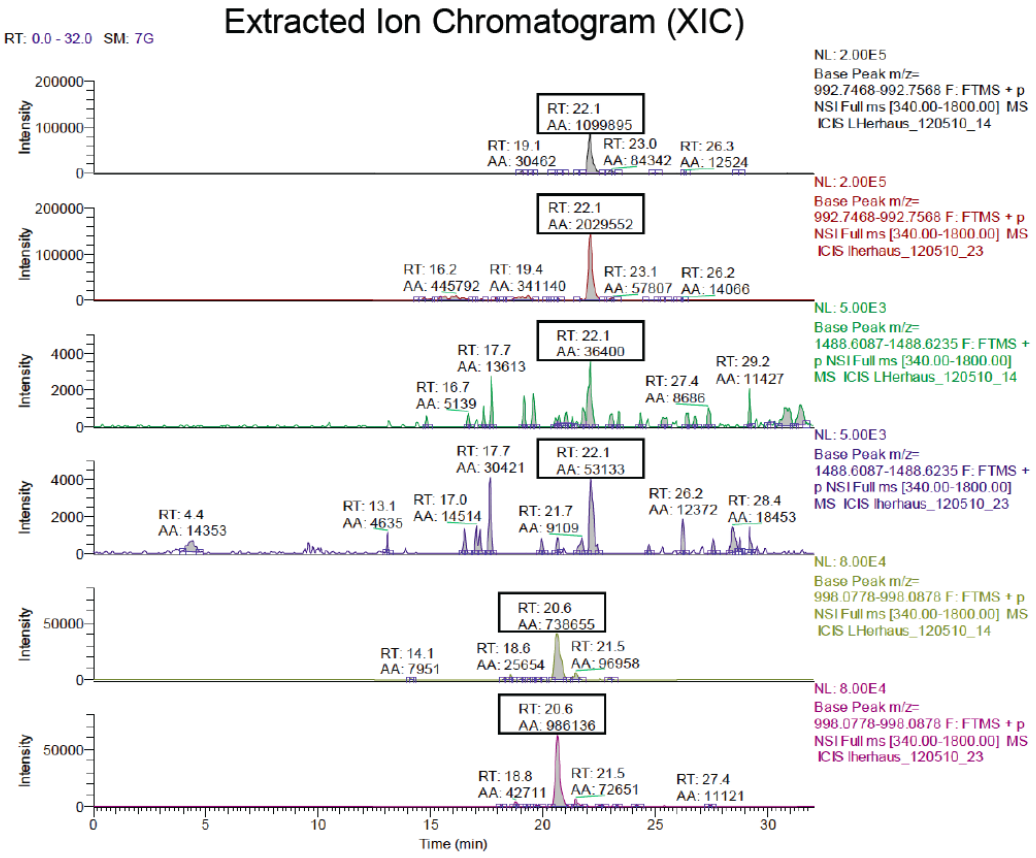

Figure S1: ALK5 binds OTUB1 but does not induce Ser[16] phosphorylation in cells. (A) Identification of the OTUB1 phospho-peptide by mass spectrometry. GFP-OTUB1 expressed in HEK293 cells treated with or without TGFβ (50 pM**,** 1 hour) was affinity-purified, resolved by SDS-PAGE, subjected to tryptic cleavage and the resulting peptides assessed by mass spectrometry and extracted ion chromatogram (XIC) analysis for detection of post-translational modifications. The Mascot and XIC analyses are indicated. Detailed protocol, analyses and the raw mass spectrometric data are included in the PRIDE database as noted in the main manuscript. (*Cont'd next page*).

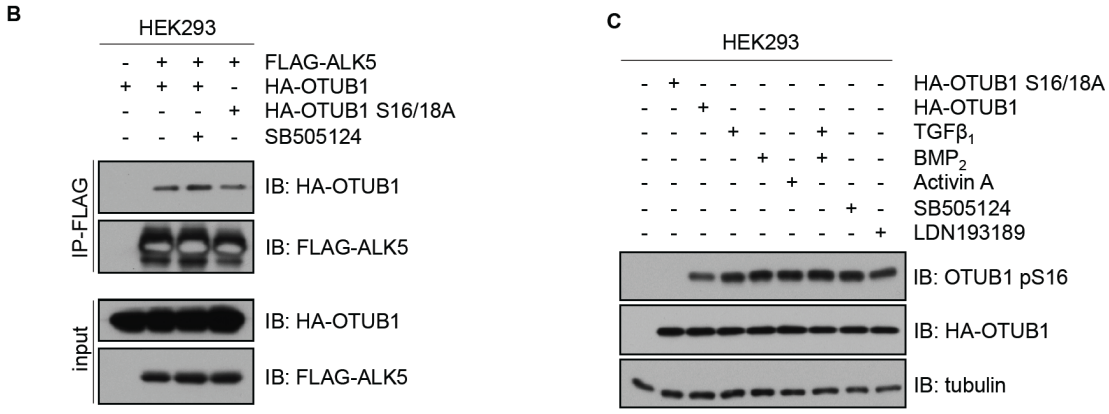

Figure S1 (cont'd): (B) HEK293 cells were co-transfected with vectors encoding N-terminal HA-tagged OTUB1 or HA-OTUB1[S16A and S18A (S16/18A)] and N-terminal FLAG-tagged ALK5. Prior to lysis cells were treated with or without SB505124 (1 μM, 1 hour). FLAG-immunoprecipitates or extracts were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. **(C)** HEK293 cells were transfected with vectors encoding N-terminal HA-tagged OTUB1 or HA-OTUB1[S16/18A]. Prior to lysis cells were treated with indicated cytokines or inhibitors [TGFβ1 (50 pM, 1 hour), BMP2 (25 ng/ml, 1 hour), Activin A (20 ng/ml, 1 hour), SB505124 (1 μM, 1 hour), LDN193189 (100 nM, 1 hour), TDB (10 μM, 4 hours)]. Extracts were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Data are representative of three independent experiments.

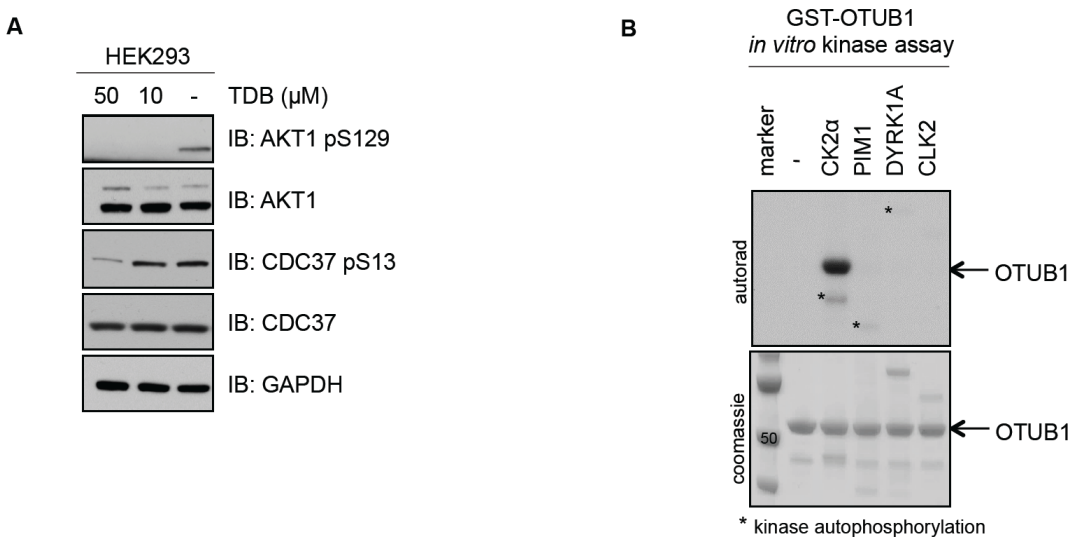

Figure S2: CK2 substrates and pSer[16]-OTUB1 phosphorylation. (A) HEK293 cells were treated with indicated amounts of TDB for 4 hours. Extracts were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. **(B)** An in vitro kinase assay was set up with different kinases (that are inhibited with TDB) using GST-OTUB1 as substrate in the presence of γ^{32}P-ATP (500 cpm/pmole). The reaction was stopped after 30 min at 30°C and the samples were resolved by SDS-PAGE, the gel was Coomassie stained and radioactivity was analyzed by autoradiography. Data are representative of of three independent experiments.

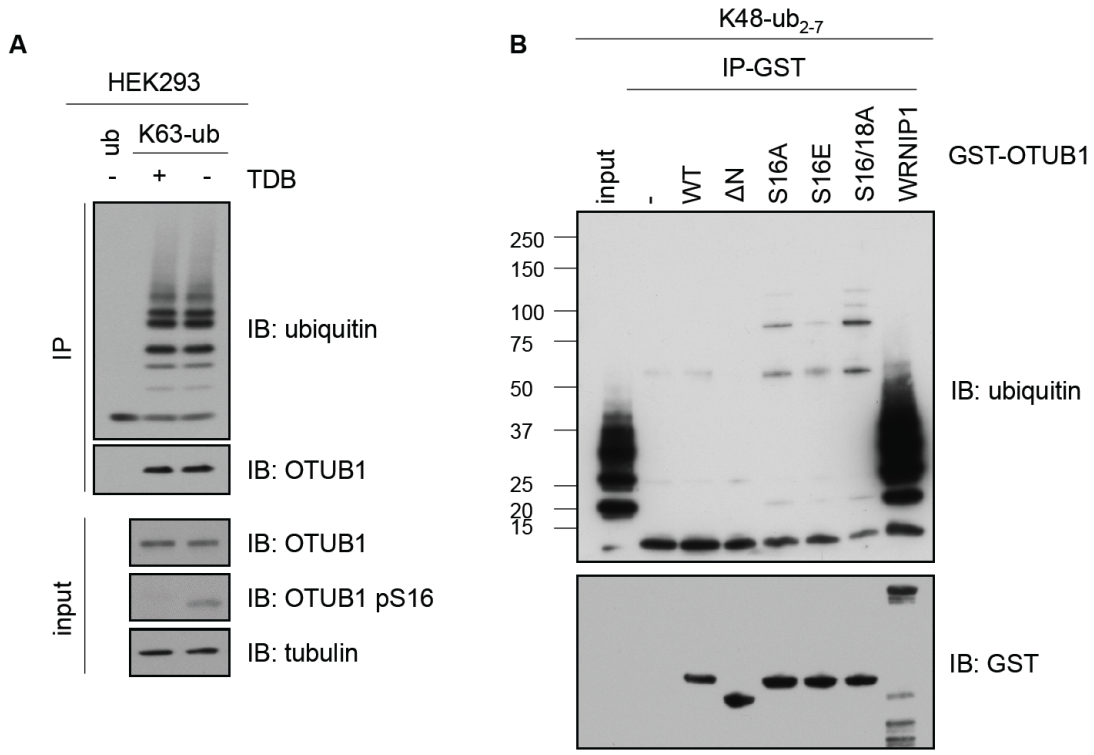

Figure S3: Interaction between OTUB1 and polyubiquitin chains. (A) K63-linked polyubiquitin chains pull-down endogenous OTUB1. HEK293 cells were treated with DMSO control or TDB (4 hours, 10 μM) and lysed. Agarose beads that were coupled to monoubiquitin (first lane, negative control) or K63-linked polyubiquitin chains were incubated with indicated extracts. The beads were washed and resolved by SDS-PAGE and processed for immunoblotting with the indicated antibodies. Extracts (20 μg protein) were also resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. **(B)** GST-OTUB1 wild type or indicated mutants and GST-WRNIP1 were incubated with K48-linked polyubiquitin chains for 1 hour at 30°C and GST-tagged proteins pulled-down with GSH-sepharose beads. The pulldowns were washed and resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Data are representative of three independent experiments.

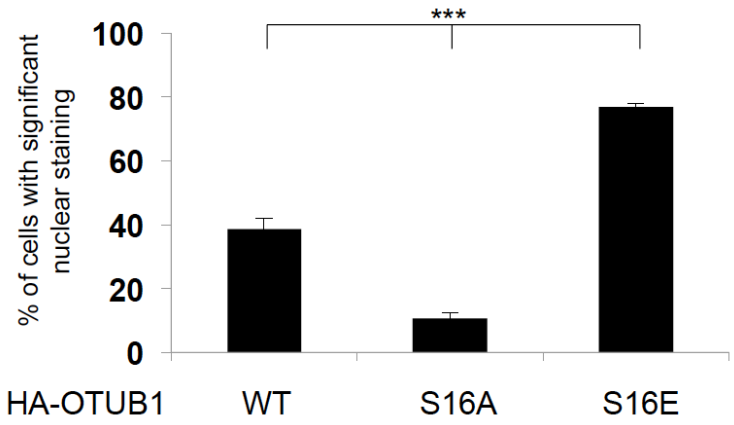

Figure S4: Quantification of Fig. 5B. The number of HA-OTUB1 [WT, S16A, S16E] cells (Figure 5B) with substantial anti-HA staining in the nucleus. Data mean ± S.D. from 3 experiments, 100 cells each. ***p<0.001.

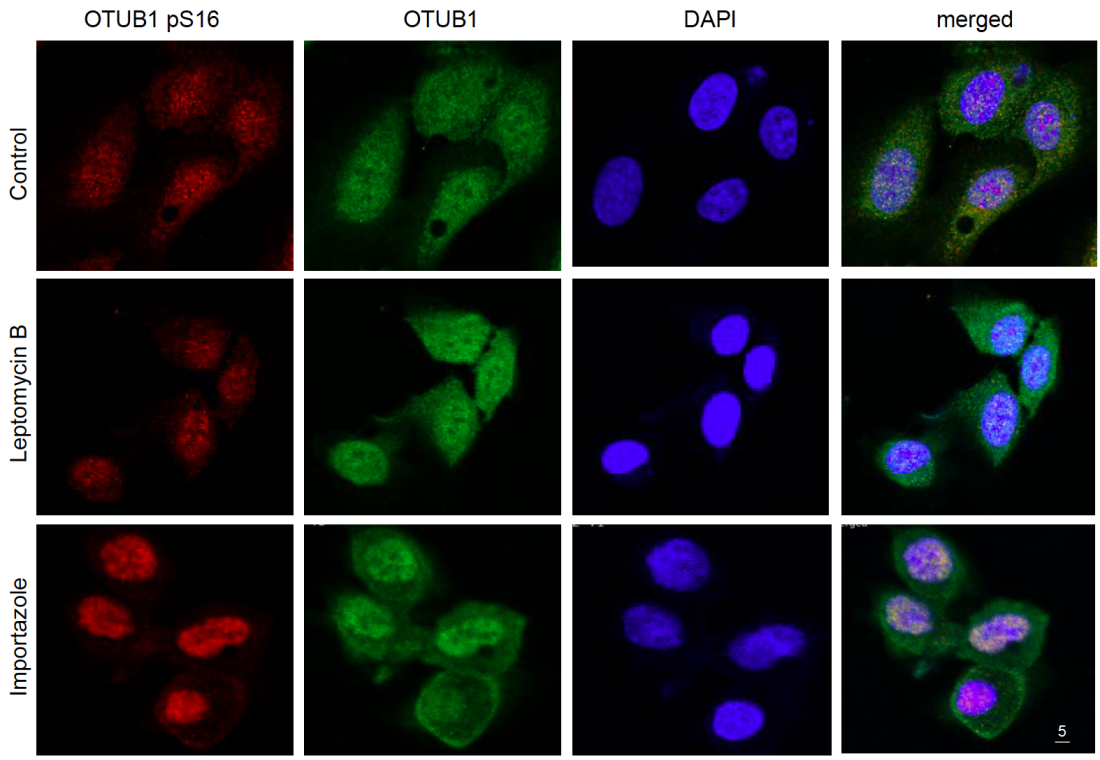

Figure S5: Effects of leptomycin B and importazole on OTUB1 localization. Fixed cell immunofluorescence with the indicated antibodies was performed on U2OS cells treated or not with leptomycin B (10 μM, 4 hours) or importazole (40 μM, 4 hours). Individual and merged pictures show pSer16-OTUB1 in red, total OTUB1 in green and DAPI in blue. Scale bar, 5 μm. Data are representative of three independent experiments.