Thompson et al., http://www.jcb.org/cgi/content/full/jcb.200909067/DC1

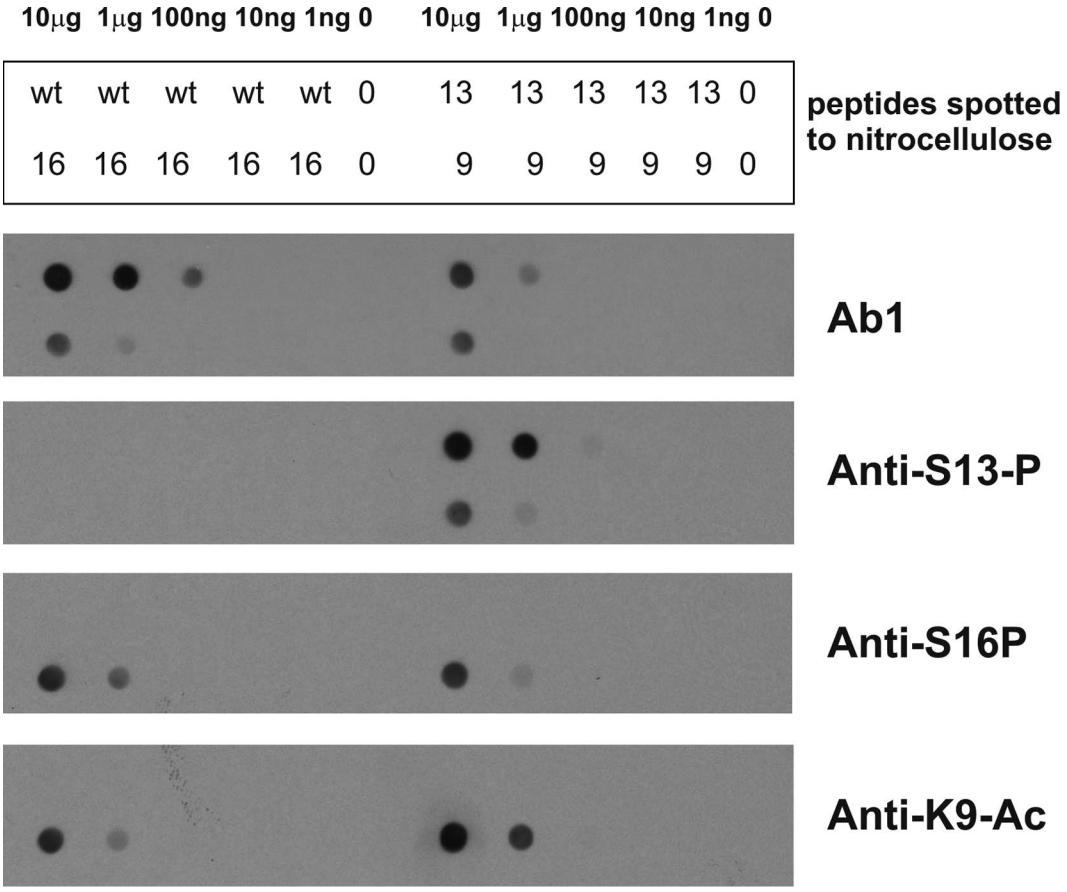

Figure S1. **Modification-specific antibodies do not detect unmodified 1–17 Htt peptide.** To verify that the three modification-specific affinity-purified rabbit polyclonal antibodies specifically recognize the modified peptides against which they were raised—p13, p16 and p13,p16,Ac9—peptides were dot-blotted to nitrocellulose with tenfold dilutions starting at 10 µg, and reactivity was compared with the control, unmodified peptide. Peptides: wt = Ac-ATLEKLMKAFESLKSF(Ahx)C-amide; 16 = CMATLEKLMKAFESLK(pS)F-amide; 13 = CMATLEKLMKAFE(pS)LKSF-amide; 9 = Ac-CLEKLM(Ac-K)AFE(pS)LK(pS)F-amide. Modification-specific and control antibodies were used to detect the peptides by Western blot. Antibodies include: (1) Ab1 generated against wt 1–17aa Htt peptide (a gift from M. DiFiglia, Harvard University, Cambridge, MA). This antibody recognizes all four peptides, but prefers the wt peptide by at least 1 order of magnitude. (2) Anti–S13-P generated against peptide 13. Anti–S13-P recognizes peptides containing phosphorylated S13 (peptide 13 and peptide 9), preferring peptide 13 to peptide 9 by over 1 order of magnitude. There is no detectable binding to unmodified wt peptide. (3) Anti–S16-P generated against peptide 16. Anti–S16-P recognizes only peptides containing phosphorylated S16 (peptide 16 and peptide 9) and did not detect unmodified wt peptide. (4) Anti–K9-Ac generated against peptide 9. Anti–K9-Ac recognizes peptide 9 and peptide 16, both containing phosphorylated S16, but prefers peptide 9 (also containing phosphorylated 13 and acetylated 9) by an approximate order of magnitude, and did not detect unmodified wt peptide.

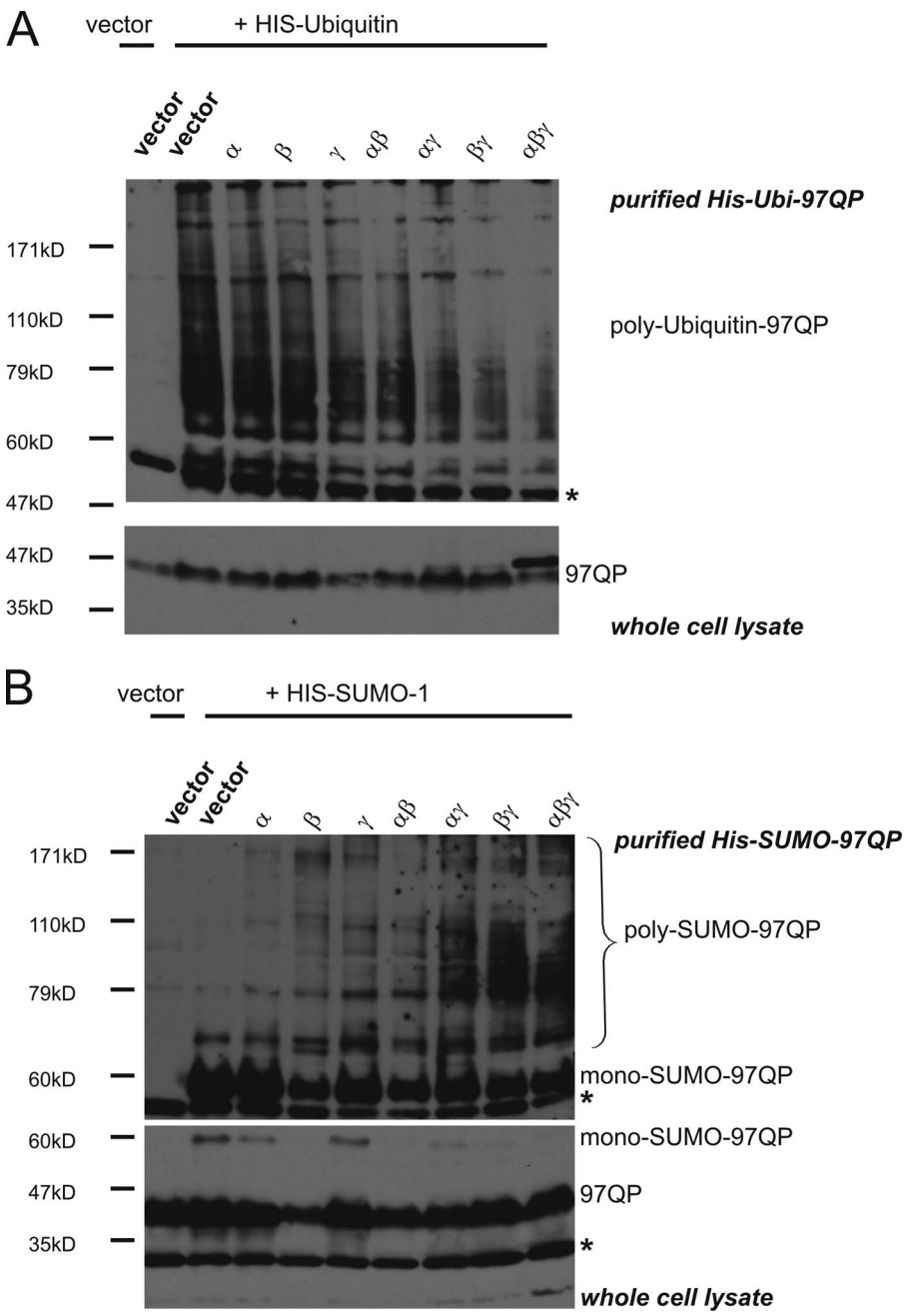

Figure S2. **IKK regulates ubiquitination and SUMOylation of Httex1p.** (A) Overexpression of IKK-αβγ reduces polyubiquitination of 97QP Httex1p. St12.7 cells were transiently transfected with vector or HIS-ubiquitin, 97QP GS, and vector or IKK subunits. Purified HIS-ubiquitin conjugates and whole-cell lysate were subjected to Western analysis with anti-Htt CAG53b. Asterisk denotes a nonspecific band. (B) Overexpression of IKK-αβγ reduces mono-SUMOylation but increases poly-SUMOylation of 97QP Httex1p. HeLa cells were transiently transfected with vector or HIS-SUMO-1, 97QP GS, and vector or IKK subunits. Purified HIS-SUMO conjugates and whole-cell lysate were subjected to Western analysis with anti-Htt CAG53b. Asterisk denotes a nonspecific band.

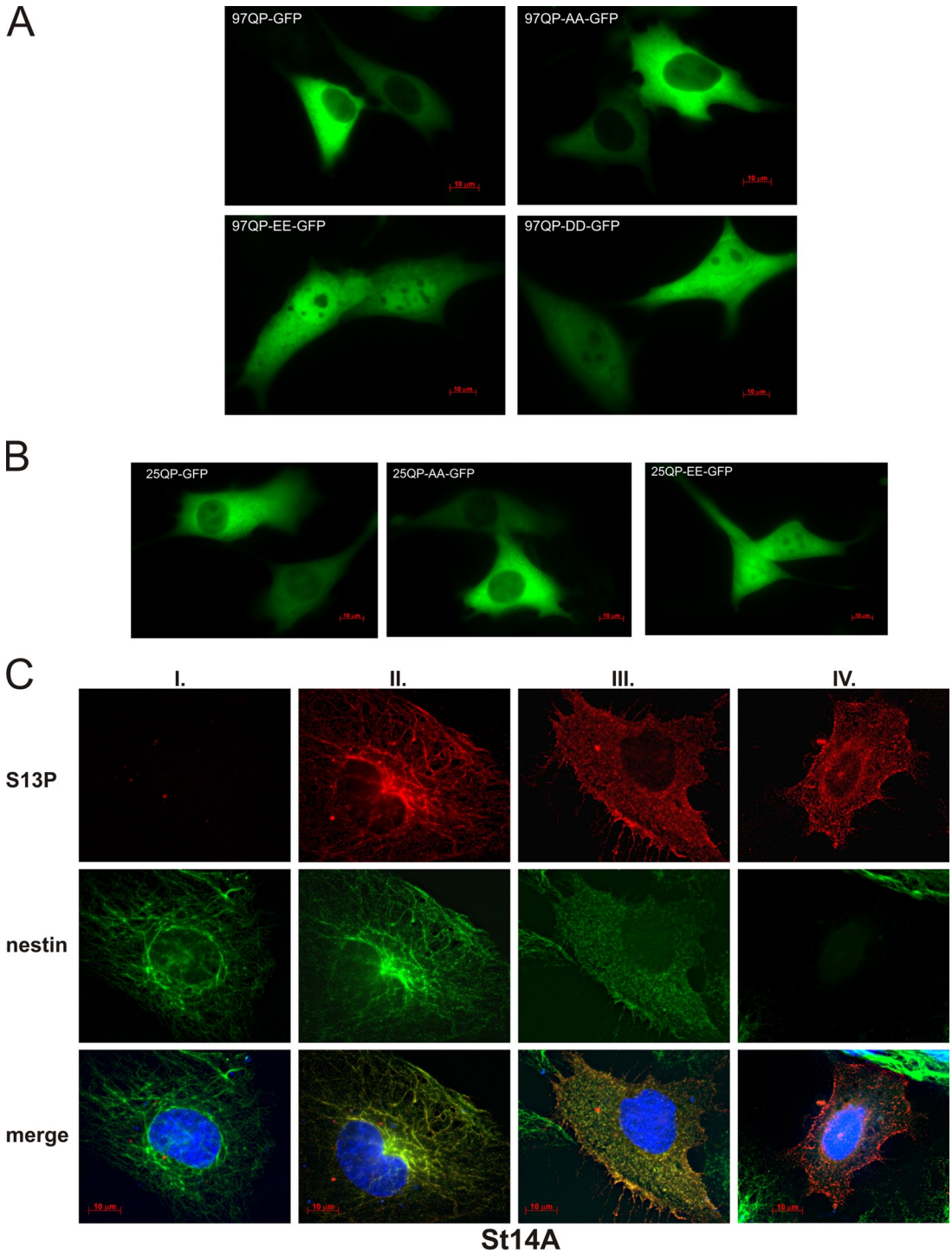

Figure S3. **Localization of phosphorylated Htt in cell culture.** (A) Mimicking phosphorylation of S13 and S16 increases 97QP-GFP nuclear localization in cell culture. 3T3 cells were transiently transfected with Httex1p constructs containing 97QP with a C-terminal GFP fusion, either control or with the following mutations: S13,16A (AA) to block phosphorylation, or S13,16E (EE) or S13,16D (DD) to mimic phosphorylation, and fluorescence microscopy was performed. (B) Mimicking phosphorylation increases nuclear targeting of unexpanded polyQ Httex1p. 3T3 cells were transiently transfected with 25QP-GFP, 25QP-AA-GFP, or 25QP-EE-GFP and fluorescence microscopy was performed. (C) S13-phosphorylated Htt colocalizes with the intermediate filament protein nestin in rat St14A cells lipofected with FLAG-IKK-β. Immunofluorescence analysis was performed using anti–S13-P and an anti–rabbit secondary antibody conjugated with Cy3 to detect endogenous wt phosphorylated Htt. Transiently transfected FLAG-IKK-β was detected with mouse anti-FLAG antibody, and endogenous nestin with mouse anti-nestin, both with a secondary anti–mouse antibody conjugated with FITC. Nuclei were detected with DAPI stain and AxioVision software (Carl Zeiss, Inc.) was used to generate 3D deconvoluted images. Approximately equal numbers of cells show filamentous staining (II) as show punctuate staining (III) for colocalized phosphorylated Htt and nestin using immunofluorescence analysis. Phosphorylated Htt and nestin can also be found in the absence of one another in FLAG-IKK-β transfected cells (I and IV). In cells displaying S13 antibody staining in the presence of exogenous IKK-β, several different types of localization patterns emerged, including strong colocalization with the intermediate filament protein nestin in both filamentous as well as punctuate structures; S13 phosphorylated Htt is detected in the cytosol in punctuate structures located both in the perinuclear region, the typical location of lysosomes, and in close proximity to the plasma membrane, suggestive of some association with the endocytic compartment. The colocalization of phosphorylated Htt in the cytoplasm with nestin could be involved in regulating protein degradation, as intermediate filaments have been found to interact with the endo-lysosomal sorting machinery (Styers et al., 2004).

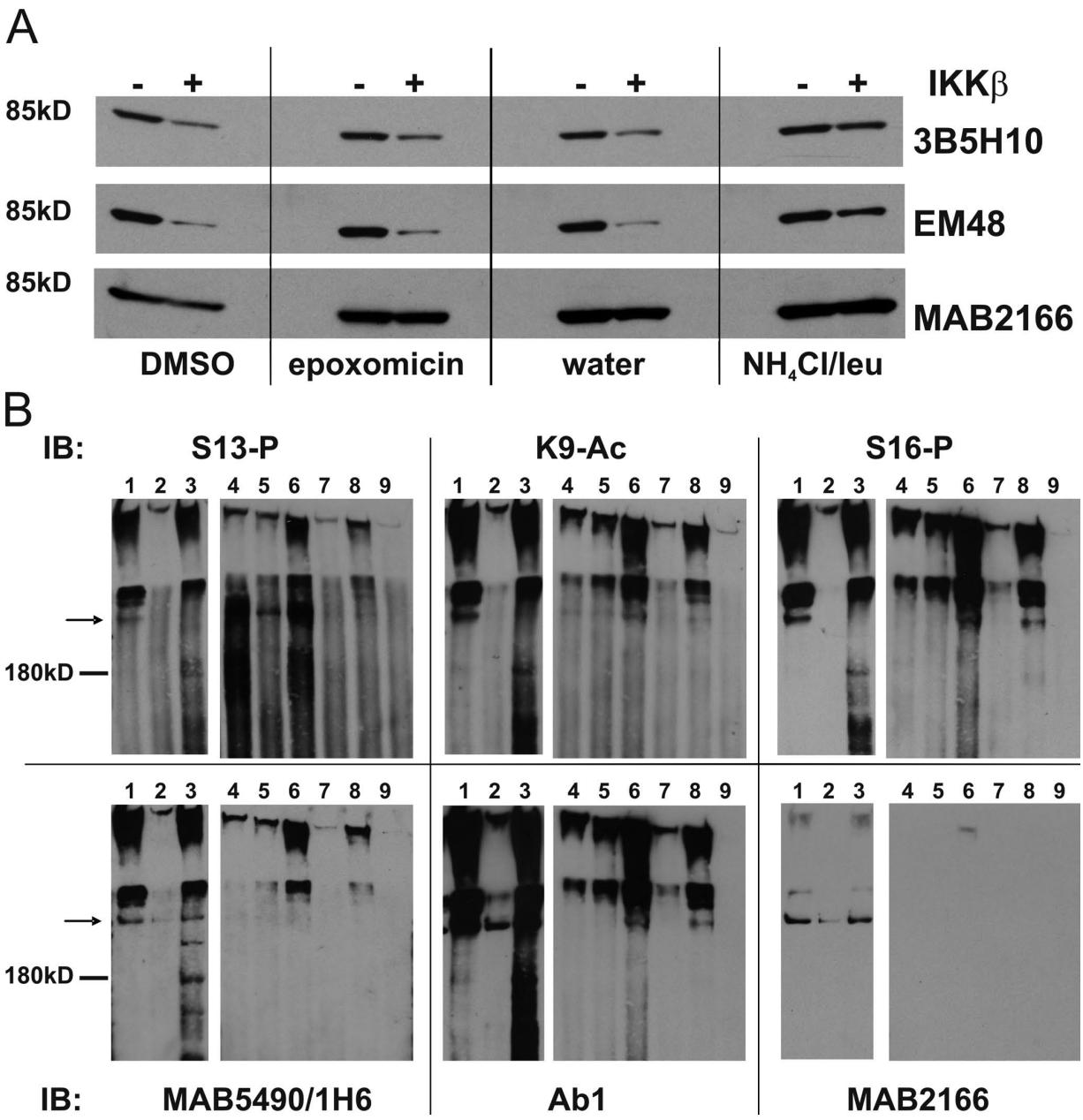

Figure S4. **Anti-Htt antibody MAB2166 may not recognize the species of Htt targeted for clearance by IKK-mediated phosphorylation.** (A) Antibodies 3B5H10 and EM48 detect Htt clearance, whereas MAB2166 does not. 15Q 586 aa Htt levels are reduced with overexpression of IKK-β as detected with 3B5H10 anti-Htt antibody (Fig. 3 D). When this same Western blot (panels showing 15Q 586 aa Htt) is probed with anti-Htt EM48, Htt levels are also reduced with IKK-β overexpression. In contrast, MAB2166 does not show this reduction well. (B) Phosphorylated and acetylated Htt species are recognized by total anti-Htt antibodies PW0595 (Enzo Life Sciences, Inc.), Ab1, CAG53b and MAB5490/1H6, but not by MAB2166. St14A cells were transiently transfected with IKK-β, and 48 h later lysed in native buffer A and centrifuged. For immunoprecipitation, 1.2 mg of supernatant was incubated for 3 h with the following antibodies: (1) PW0595; (2) Ab1; (3) CAG53b; (4) anti–S13-P; (5) anti–K9-Ac; (6) JG1; (7) anti–S16-P; (8) anti–T3-P; and (9) buffer control. Antibodies were purified on Protein G-Plus Agarose (Santa Cruz Biotechnology, Inc.), washed three times in native buffer A, and subjected to Western analysis with anti-Htt antibodies anti–S13-P, anti–K9-Ac, anti–S16-P, MAB5490/1H6, Ab1, and MAB2166. Immunoprecipitated endogenous wt Htt is present as an insoluble, aggregated species in the stacking gel and at the top of the separating gel, as a soluble form at the standard 350-kD size marked with an arrow, and as higher molecular weight soluble species, which may indicate its post-translational modification. The phospho- and acetyl-specific antibodies all recognize the insoluble Htt species, whereas MAB2166 primarily detects the 350-kD band.

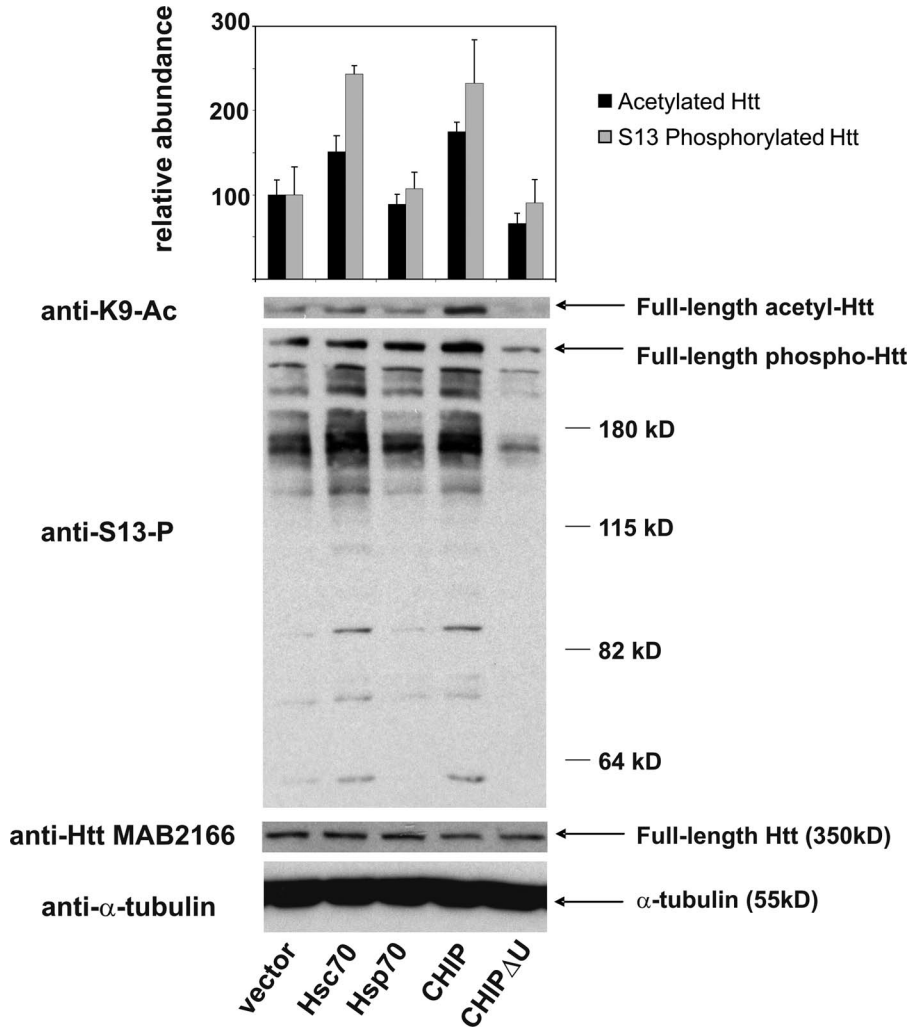

Figure S5. **Hsc70 modulates levels of endogenous phosphorylated and acetylated Htt in cell culture.** Hsc70 and CHIP increase levels of endogenous wt acetylated and phosphorylated Htt in cells overexpressing IKK-β. St14A cells were transiently cotransfected with vector control, Hsc70, Hsp70, CHIP, or CHIP with a deletion of its Ubox domain (CHIPΔU) together with IKK-β. Cells were lysed in native lysis buffer A. Levels of phosphorylated and acetylated Htt were detected on Western blot with anti–S13-P and anti–K9-Ac antibodies, and unmodified Htt was detected with anti-Htt MAB2166. Equal loading of protein is shown by levels of α-tubulin detected with anti–α-tubulin antibody.

References

Styers, M.L., G. Salazar, R. Love, A.A. Peden, A.P. Kowalczyk, and V. Faundez. 2004. The endo-lysosomal sorting machinery interacts with the intermediate filament cytoskeleton. *Mol. Biol. Cell.* 15:5369–5382. doi:10.1091/mbc.E04-03-0272