**Supplemental Information**

**Force-induced dephosphorylation activates**

**the cochaperone BAG3 to coordinate**

**protein homeostasis and membrane traffic**

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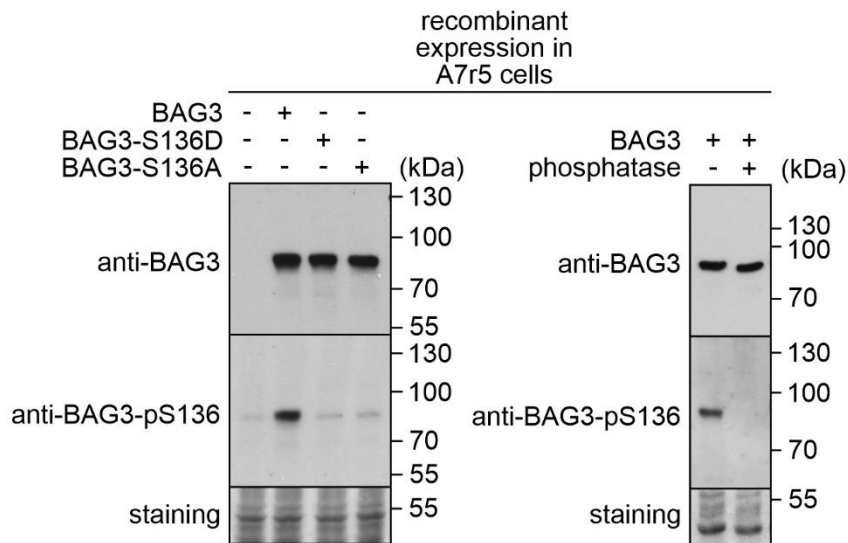
**Figure S1. The anti-BAG3-pS136 antibody specifically recognizes BAG3 upon phosphorylation of serine-136. Related to Figure 1.**

A7r5 cells were transiently transfected with plasmids encoding BAG3, BAG3-S136D, and BAG3-S136A. 48 hours after transfection cells were collected and lysates were analysed by Western blotting using an anti-BAG3 and an anti-BAG3-pS136 antibody. While the anti-BAG3-pS136 antibody recognized wild-type BAG3, it did not react with the mutant variants, in which S136 was exchanged to alanine or aspartic acid. Moreover, BAG3 was no longer recognized by the anti-BAG3-pS136 antibody when lysates were treated with alkaline phosphatase prior to Western blotting. Staining corresponds to Ponceau S staining.
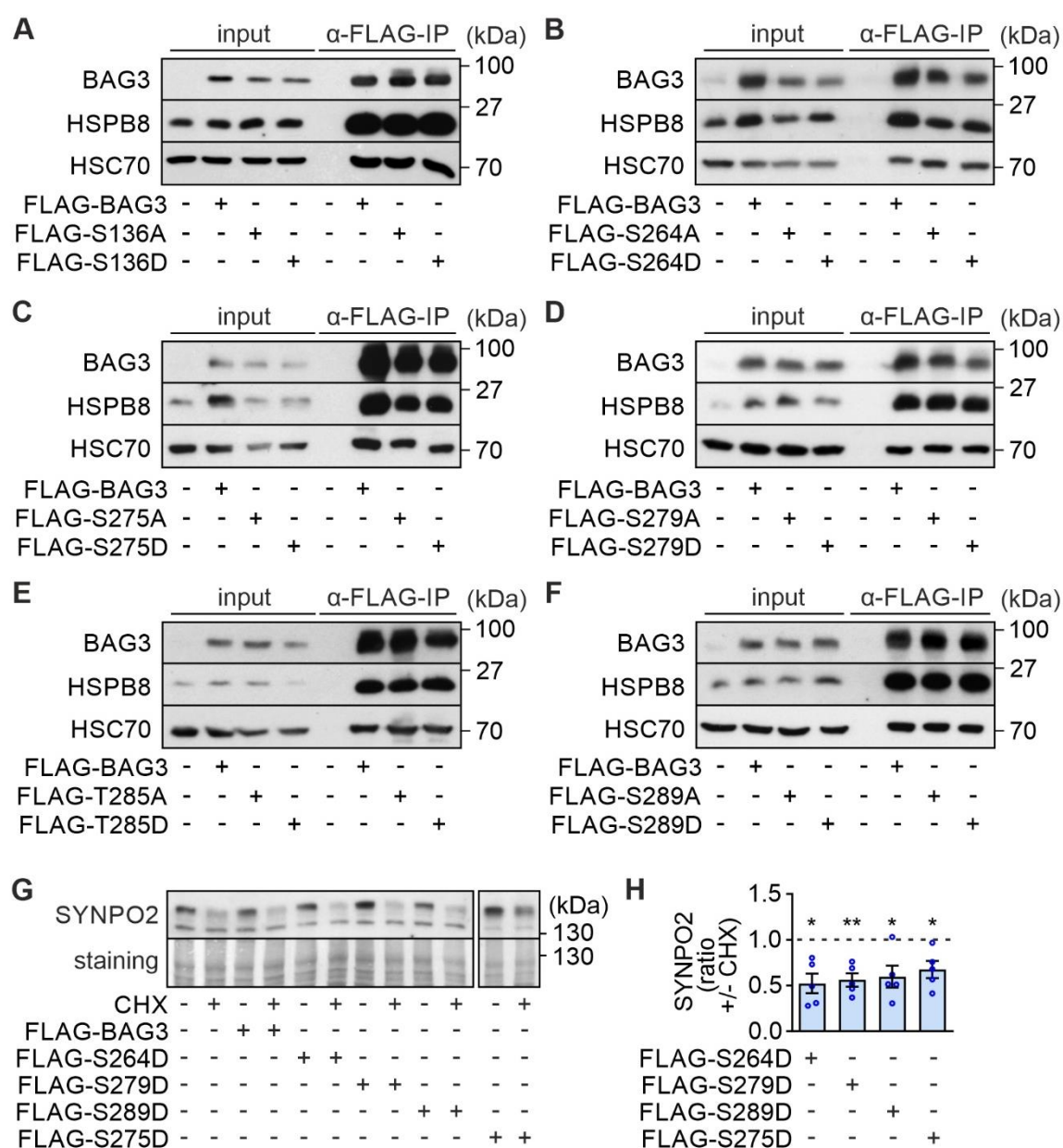
**Figure S2. Impact of phosphorylation mimicry of force regulated phosphosites of BAG3 on CASA complex formation and CASA activity. Related to Figure 2.**

(A-F) Phosphorylation mimicry on S136, S264, S275, S279, T285, and S289 of BAG3 does not affect CASA complex formation. Phosphosite mutant variants of BAG3 were transiently expressed in HeLa cells as FLAG-epitope tagged proteins followed by anti-FLAG immunoprecipitation. Immunoprecipitated complexes were probed with specific antibodies for the presence of BAG3, HSPB8, and HSC70.

(G) Phosphorylation mimicry on S264, S275, S279, and S289 of BAG3 does not abrogate CASA activity. BAG3 and BAG3 phosphosite mutant variants were transiently

expressed in A7r5 rat smooth muscle cells. 48 hours after transfection, cells were treated with cycloheximide (CHX) for 3 hours to block protein synthesis. Lysates of A7r5 cells that recombinantly express the indicated proteins were prepared and subjected to Western blot analysis using an anti-SYNPO2 antibodies.

(H) Levels of SYNPO2, obtained under (G), were quantified, levels detected in the absence of CHX were set to 1 for each transfectant, and the ratios of levels with and without CHX treatment were determined. The dashed line indicates a ratio of 1 with no changes upon CHX treatment. Data are shown as mean values +/- SEM, n = 5. Quantification for cells that received the empty plasmid and for FLAG-BAG3 overexpressing cells is included in Figure 2E.

Statistical analysis was carried out using two-tailed unpaired t-test with Welch's correction: $*p < 0.05$, $**p < 0.01$.
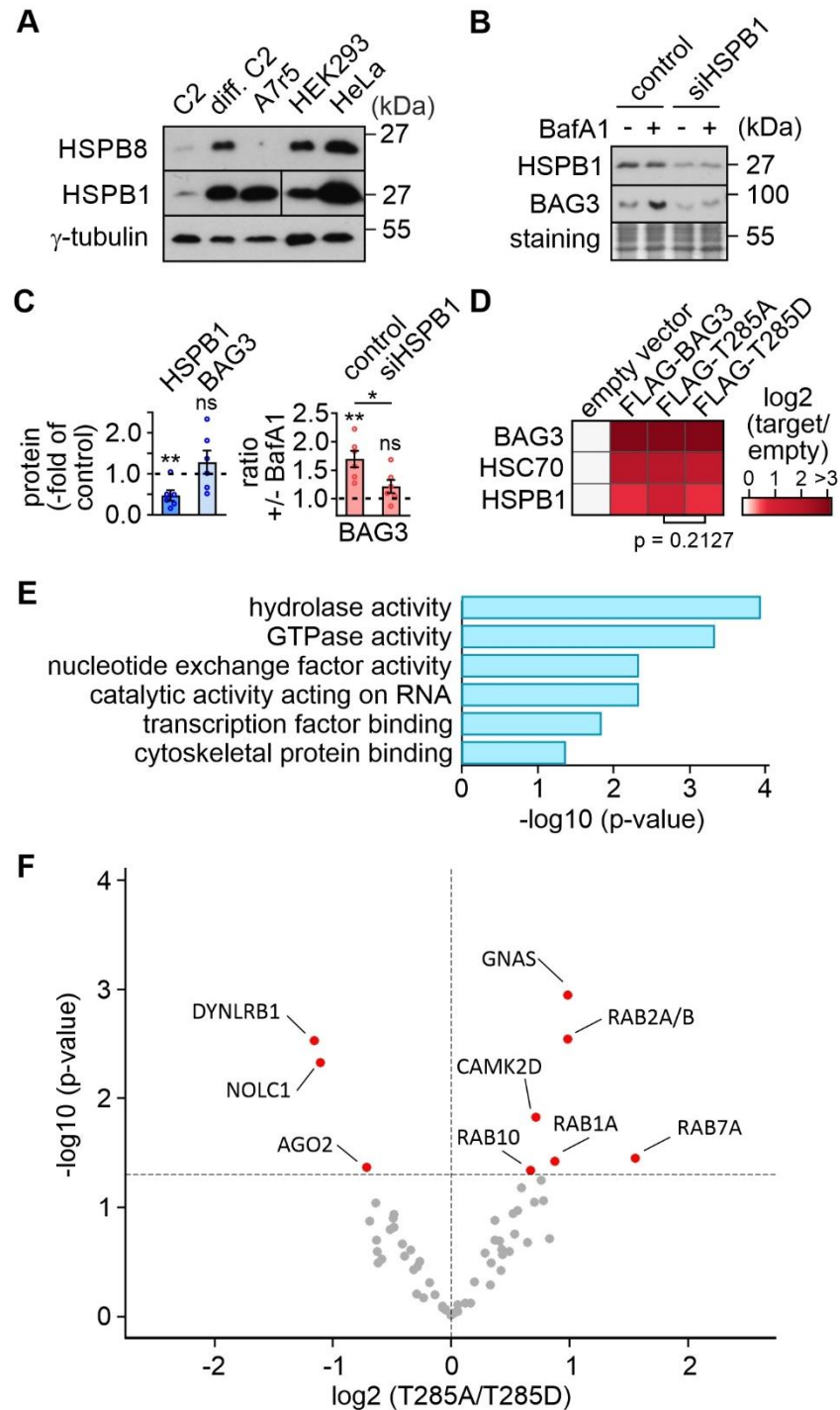
**Figure S3. Differential proteomics of BAG3-T285A and D complexes reveals RAB GTPase as dephosphorylation-dependent interactors of BAG3. Related to Figure 3.**

(A) A7r5 smooth muscle cells express the small heat shock protein and BAG3 interactor HSPB1 but not HSPB8.

(B) A7r5 cells were transfected with control siRNA (control) or HSPB1-targeting siRNA (siHSPB1). 7 hours before lysis cells were incubated with solvent (-) or the autophagy inhibitor Bafilomycin A1 (BafA1) (+). Cell lysates were analysed with specific antibodies against the indicated proteins. Equal loading of samples was verified by Ponceau S staining of the membrane. In the presence of BafA1, BAG3 accumulated in control cells but not in HSPB1 depleted cells, indicating that HSPB1 is required for CASA in A7r5 smooth muscle cells.

(C) Data obtained under (B) were quantified. Protein levels detected in control cells were set to 1, and the ratios of levels between HSPB1-depleted and control cells was calculated (blue column bars). To determine degradation rates, protein levels detected in the absence of BafA1 were set to 1, and the ratios of levels with and without BafA1 treatment were determined (light red column bars). Dashed lines indicate the control values. Data are shown as mean values +/- SEM, n = 5.

Statistical analysis was carried out using two-tailed unpaired t-test with Welch's correction: **$p < 0.01$, *$p < 0.05$.

(D) HSC70 and HSPB1 were detected in complexes of wild-type BAG3, and BAG3-T285A and D mutant variants by interaction proteomics. Heat maps show protein abundance as log2-transformed mean intensity compared to mean background intensity (FLAG-BAG3-construct/empty). See Data S2A for more information.

(E) Gene Ontology (GO) terms enriched among proteins identified as BAG3 interactors. GO terms were selected from significantly enriched categories (FDR-corrected p-value < 0.05). For GO-term ID numbers see Data S2B.

(F) Volcano plot visualizing log2 transformed ratio of mean protein abundance in pulldowns with BAG3-T285A compared to BAG3-T285D variants, plotted against -log10 (LIMMA moderated t-test p-value T285A/T285D) as a measure of significance. See Data S2C for more information.

For all panels, empty vector, n = 5; FLAG-BAG3, n = 5; FLAG-T285D, n = 5; FLAG-T285A, n = 5.
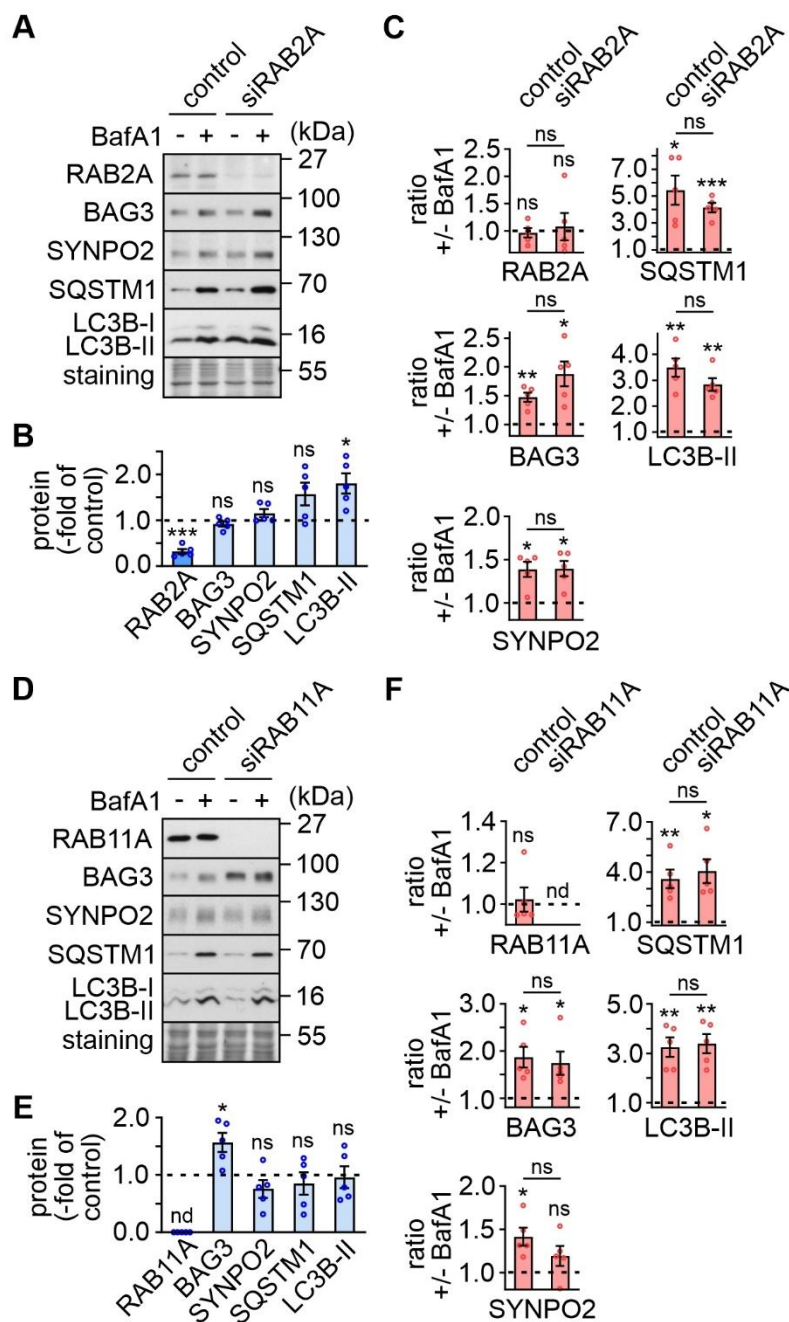
**Figure S4. RAB2A and RAB11A are dispensable for CASA in murine myotubes. Related to Figure 4.**

(A) Differentiating C2 myoblasts were transfected with control siRNA or RAB2A-targeting siRNA (siRAB2A) on day 0 and day 2 of the differentiation process. After 5 days of differentiation and 5 hours before lysis myotubes were incubated with solvent DMSO (-) or BafA1 (+). Myotube lysates were analysed with specific antibodies against the indicated proteins.

(B) RAB2A depletion leads to an increase in the steady state levels of SQSTM1, and LC3B-II. Data obtained under (A) were quantified. Protein levels observed in solvent treated and control siRNA transfected cells were set to 1 and were compared to levels in solvent treated RAB2A-depleted cells. The dashed line indicates the control value. Data are shown as mean values +/- SEM, n = 5.

(C) RAB2A depletion does not attenuate CASA. Data obtained under (A) were quantified. The ratio of protein levels with and without BafA1 treatment (ratio +/- BafA1) was determined. The dashed line indicates a ratio of 1 with no changes upon BafA1 treatment. Data represent mean values +/- SEM, n = 5.

(D) Differentiating C2 myoblasts were transfected with control siRNA or RAB11A-targeting siRNA (siRAB11A) on day 0 and day 2 of the differentiation process. After 5 days of differentiation and 5 hours before lysis myotubes were incubated with solvent DMSO (-) or BafA1 (+). Myotube lysates were analysed with specific antibodies against the indicated proteins.

(E) RAB11A depletion leads to an increase in the steady state levels of BAG3. Data obtained under (D) were quantified. Protein levels observed in solvent treated and control siRNA transfected cells were set to 1 and were compared to levels in solvent treated RAB11A-depleted cells. The dashed line indicates the control value. Data are shown as mean values +/- SEM, n = 5.

(F) RAB11A depletion does not attenuate CASA. Data obtained under (D) were quantified. The ratio of protein levels with and without BafA1 treatment (ratio +/- BafA1) was determined. The dashed line indicates a ratio of 1 with no changes upon BafA1 treatment. Data are shown as mean values +/- SEM, n = 5.

Statistical analysis was carried out using two-tailed unpaired t-test with Welch's correction: *p < 0.05, **p < 0.01, ***p < 0.001, ns – non-significant, nd – non-detectable. Asterisks above column bars indicate significance compared to control. Asterisks above column-connecting lines indicate significance between compared samples.
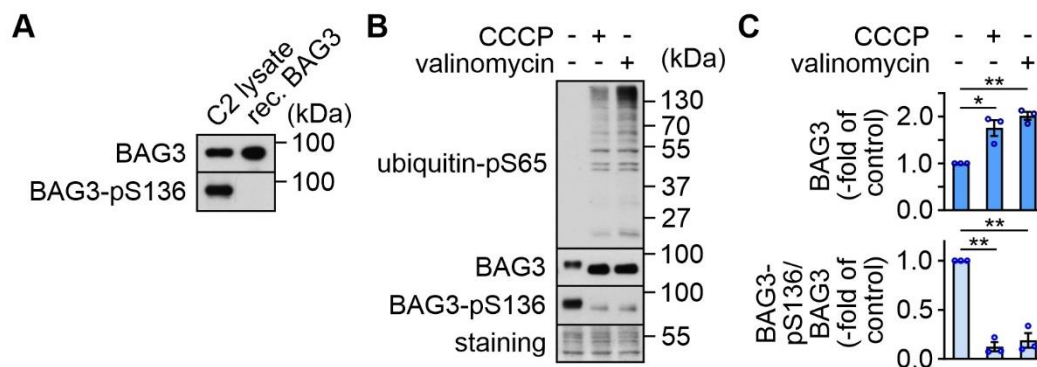
**Figure S5. BAG3 dephosphorylation is triggered by pharmacological induction of mitophagy. Related to Figure 5.**

(A) A lysate of C2 myotubes (C2 lysate, 40 µg of protein) and bacterially expressed, recombinant and purified BAG3 (rec. BAG3, 12 ng) were separated by SDS-PAGE and analysed by immunoblotting with an anti-BAG3 antibody or the phosphospecific antibody anti-BAG3-pS136 as indicated. The recombinant protein does not display the phosphorylation characteristic for inactivated BAG3 in mammalian cells.

(B) C2 myotubes were treated with the mitophagy-inducing compounds CCCP and valinomycin for 16 hours. Control cells received the same amount of solvent. Afterwards, cells were collected, lysed, and analysed by immunoblotting with antibodies against the indicated proteins.

(C) Protein levels determined under (B) were quantified. Levels of BAG3 detected in control cells were set to 1 and fold changes in the presence of CCCP and valinomycin were calculated (dark blue columns). In addition, the ratio of BAG3-pS136 to total BAG3 was calculated with the value obtained for control cells set to 1. Data represent mean values +/- SEM, n = 3.

Statistical analysis was carried out using two-tailed unpaired t-test with Welch's correction: *p < 0.05, **p < 0.01.