BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins

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Supplementary Information

Methods

Plasmids.

Expression plasmids for untagged human BAG3 (pBAG3-N1), human BAG3 fused to EGFP (pBAG3.EGFP-N1), EGFP-tagged SOD1 constructs (SOD^{G85R}-GFP and SOD^{WT}-GFP) and HA fused to ubiquitin (HA-Ubiquitin) were described previously (Gamerdinger et al, 2009; Lim et al, 2005; Witan et al, 2008).

FLAG-tagged BAG3 (FLAG-BAG3) and FLAG-tagged BAG3 deletion mutants (FLAG-BAG-domain and FLAG-PxxP-BAG-domain) were constructed by PCR amplification of corresponding DNA fragments using pBAG3-N1 as a template. HA-tagged Hsp70 (HA-Hsp70) and FLAG-tagged p50 (FLAG-p50) DNA fragments were obtained by PCR amplification using pEGFP-Hsp70 (Zeng et al, 2004) and cDNA from HEK cells, respectively, as templates. All DNA fragments were inserted into pEGFP-N1 vector (Clontech, Mountain View, CA, USA), in which the EGFP gene was removed.

The FLAG-tagged BAG3 deletion construct lacking the BAG domain (amino acids 421-498; FLAG-(Δ421-498)-BAG3) was cloned using the Quikchange lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and FLAG-BAG3 as a template.

The GST plasmid was obtained by cloning the GST gene from pGEX (GE Healthcare, Munich, Germany) into the pEF-Bos-ex vector (Murai et al, 1998). To obtain the GST-DIC plasmid, the dynein intermediate chain 1 (transcript variant 2) CDS was amplified by PCR using HEK cDNA as a template and cloned into the GST plasmid.

Cell culture

Human embryonic kidney 293 cells (HEK, 293), African green monkey kidney Cos7 cells and primary human fibroblasts (IMR90) were cultured in Dulbecco modified Eagle medium supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum, 1 × nonessential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a 5% CO^2-humidified atmosphere.

Dynein GST pull-down analysis.

The interaction of cytoplasmic proteins with the dynein motor complex was analyzed as previously described (Ström et al, 2008) with some modifications. In brief, cells transfected with GST or GST-DIC were lysed in RIPA buffer (50 mM Tris-HCl pH: 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 0.1% SDS) for 15 min on ice. Cell lysates were cleared by centrifugation at 1,000 × g for 10 min at 4°C. For GST pull-down, 400 µl of lysate (~1 µg/µl protein) were incubated with 50 µl of a 50% slurry of glutathione-Sepharose 4B (GE Healthcare, Munich, Germany) in low-retention tubes (Kisker-Biotech, Steinfurt, Germany) for 2 h at 4°C with end-over-end rotation. The glutathione beads were then collected by centrifugation at 500 × g for 5 min at 4°C and washed two times with ice-cold RIPA and one time with RIPA lacking SDS. The bound proteins were then released by boiling for 5 min at 99°C in 2x SDS-PAGE loading buffer (10% SDS, 20% glycerine, 125 mM Tris, 1 mM EDTA, 0.002% bromphenol blue and 10% β-mercaptoethanol) for subsequent immunoblot analysis.

Sucrose density gradient analysis.

For small scale sucrose density gradient analysis, cells were lysed on ice for 20 min in NP40 buffer (25 mM HEPES-KOH pH: 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5 mM DTT, 1% protease inhibitor mix, 1% phosphatase inhibitor mix). After centrifugation (18.000 x g, 30 min, 4°C) 100 µl of the supernatant (200 µg total protein) were loaded on the top of a 2 ml linear 5-20% sucrose gradient prepared in a buffer containing 25 mM HEPES-KOH pH: 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, 0.5 mM DTT. After ultracentrifugation (55.000 rpm) in a TLS-55 rotor (BeckmannCoulter, Krefeld, Germany) for 2 h at 4°C, 14 fractions of 150 µl were collected beginning at the top of the gradient. Subsequently, 10 and 50 µl of each fraction were directly subjected to immunoblot and GST pull-down analysis, respectively. Detection of endogenous 20S proteasome complex served as 20S migration marker on sucrose density gradients.

Immunohistochemistry.
Spinal cords from 4% PFA-perfused mice were cut on a cryostat (Leica microsystems, Wetzlar, Germany) to 10-15 µm thick sections. Prior to immunostaining, free floating sections were quenched as previously described (Sun et al, 2002) to reduce autofluorescence. For immunostaining sections were incubated for 1-2 h at RT in PBS/0.5% Triton-X100 (PBS-T) containing 3% bovine serum albumin to block unspecific antibody binding sites. Sections were then incubated with BAG3 antibody (Proteintech Group, Chicago, IL, USA) in PBS-T overnight at 4°C with gentle agitation. After washing two times with PBS-T, sections were incubated with Cy3-conjugated secondary antibody (donkey anti-rabbit, Jackson Immunoresearch, Newmarket, UK) in PBS-T for 2 h at RT. The sections were then washed two times with PBS-T and incubated for 2 h at RT with 1 µg/µl rabbit IgG antibody solution (Sigma, Schnelldorf, Germany) to block free binding domains of the secondary antibody. Subsequently, sections were incubated with rabbit monoclonal SOD1 (Epitomics, Burlingame, CA, USA) coupled to DyeLight NHS649 (Thermo Scientific, Bonn, Germany) in PBS-T for 2 h at RT. After washing two times with PBS-T, sections were mounted onto glass slides with ProLong antifade reagent (Invitrogen, Darmstadt, Germany) and visualized on a confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Antibodies	**Host**	**Supplier**
FLAG	rabbit	Sigma, F7425
FLAG	mouse	Sigma, F3165
HA	rabbit	Sigma, H6908
BAG1 (cBAG)	rabbit	Gamerdinger et al., 2009
BAG2	rabbit	Epitomics, 2610-1
BAG3	rabbit	Proteintech Group, 10599-1-AP
BAG5	rabbit	Novus Biologicals, NB100-56091
GST	mouse	Sigma, G1160
GFP	mouse	Covance, MMS-118P
Hsp70	mouse	Stressgen, C92F3A-5
Hsc/Hsp70	mouse	Stressgen, SPA-820
Vimentin	rabbit	Santa Cruz, sc-5565
p62/SQSTM1	mouse	Santa Cruz, sc-28359
p62/SQSTM1 (used in mouse tissue analysis)	rabbit	Sigma, P0067
NBR1	rabbit	Sigma, HPA022999
LAMP1	rat	BD Pharmingen, 553792
LAMP2	mouse	abcam, ab25631
Atg7	rabbit	Sigma, A2856
LC3	rabbit	Sigma, L7543
Dynactin ($p150^{Glued}$)	rabbit	Santa Cruz, sc-11363
Dynein (intermediate chain)	mouse	Sigma, D5167
20S proteasome subunit α1	rabbit	Santa Cruz, sc-67046
SOD1	rabbit	Epitomics, 2018-1
Ubiquitin	rabbit	Dako, Z0458
Poly-Ubiquitin (FK1)	mouse	Biomol, PW8805
Histone H3	mouse	Abcam, ab6000
Actin	rabbit	Sigma, A5060
Tubulin	mouse	Sigma, T9026
Gapdh	mouse	abcam, ab9482

Supplementary References

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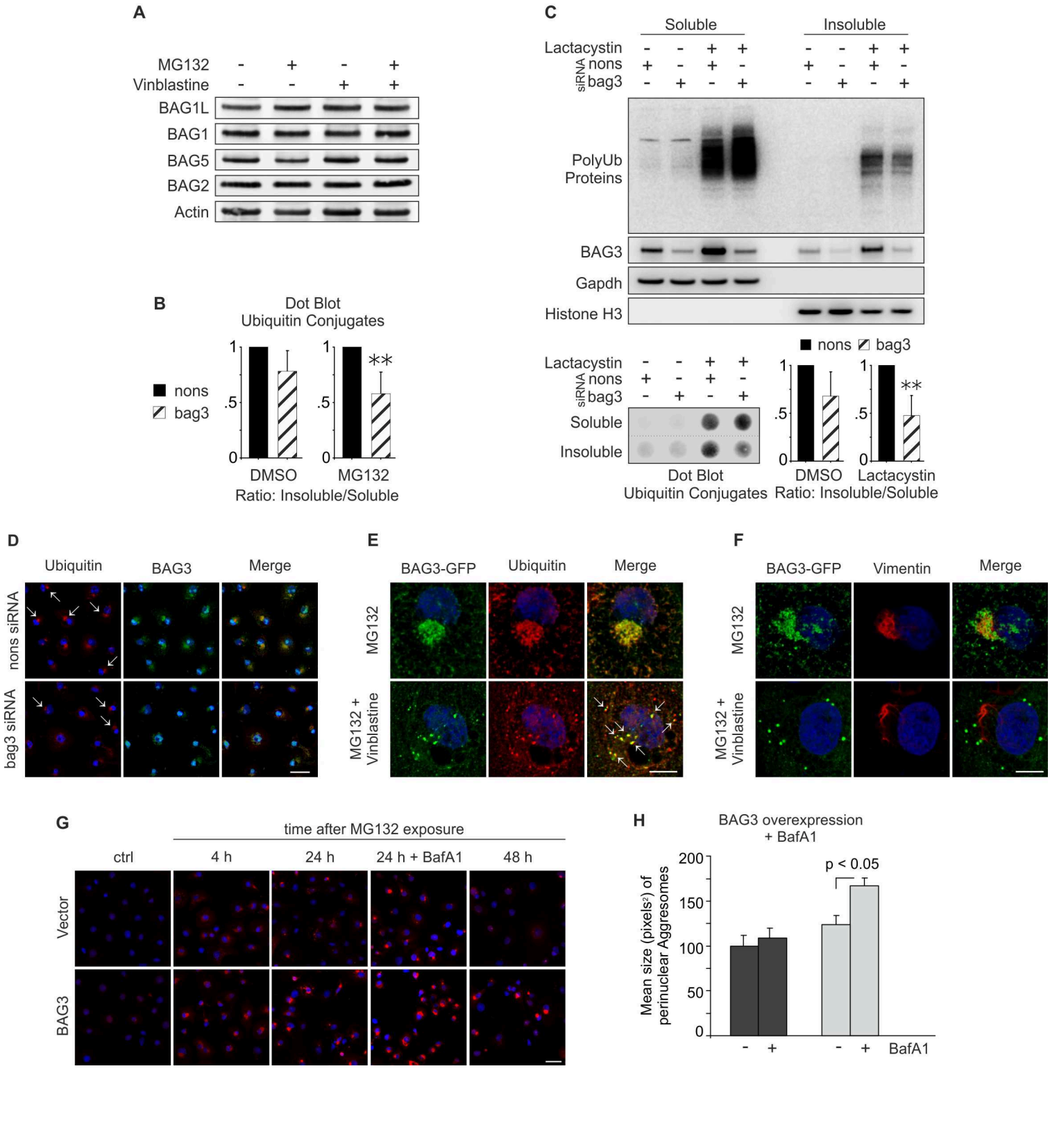
Supplementary Figure legends

Fig. S1 (**A**) Immunoblot analysis of BAG family proteins in Cos7 cells treated for 12 h with the proteasome inhibitor MG132 (25 µM) in the absence and the presence of the microtubule-depolymerizing agent vinblastine (25 µM). (**B**) Densitometric quantification of dot blot analyses as presented in Fig. 1G. Diagrams display the ratios between 0.5% NP40-insoluble and soluble ubiquitinated protein species in nonsense (nons) and bag3 siRNA transfected cells treated with MG132 (25 µM, 12 h) or DMSO as vehicle control. **p<0.01, n=3. (**C**) Same analysis as shown in Fig. 1G and S1B but with primary human IMR90 cells and the proteasome inhibitor lactacystin. (**D**) Cos7 cells were transfected for 24 h with nonsense (nons) or bag3 siRNA and treated with MG132 (25 µM, 12 h). Aggresomes were visualized by ubiquitin immunostaining. Scale Bar: 50 µm. (**E**) and (**F**) Immunofluorescence staining of ubiquitin (E) and vimentin (F) in Cos7 cells transfected with BAG3 fused to GFP (BAG3-GFP) and treated as in (A). Arrows point to ubiquitin- and BAG3-GFP-positive micro-aggregates (diameter ≤ 1 µm) dispersed throughout the cytoplasm. Sytox orange was used to stain nuclei (pseudo-colored in blue). Scale bar: 10 µm. (**G**) Representative immunofluorescence images of analysis presented in Fig. 1E, F (H). ctrl, control; BafA1, bafilomycin A1. Scale Bar: 50 µm. (**H**) Cells were treated as in (Fig 1E) but 4 h after MG132 wash out, bafilomycin A1 (BafA1, 5 µM) was added for 20 h to inhibit aggresome clearance. Diagram shows the mean size of aggresomes. Images are shown in Fig S1G.

Fig. S2 (**A**) Protein extracts from cells transfected for 18 h with GST or GST fused to dynein intermediate chain (GST-DIC) were fractionated on a 5-20% linear sucrose gradient by ultracentrifugation. The levels of endogenous dynein intermediate chain (DIC) and $p150^{Glued}$ as well as the expressed GST and GST-DIC were then examined in each collected fraction by Western-blot analysis. Migration of a 20S marker protein is indicated. (**B**) BAG3 co-immunoprecipitation (IP) analysis of Cos7 cells treated with the proteasome inhibitor MG132 (25 µM, 12 h). The obtained BAG3 immunocomplexes were analyzed for the presence of co-precipitated endogenous dynein intermediate chain (DIC). Purified rabbit (rb) IgG was used as negative control for IP. Left panel shows relative amounts of proteins in lysates used for Co-IP. (**C**) IMR90 cells were transfected with FLAG-tagged full-length BAG3 (FLAG-BAG3) or FLAG-tagged BAG deletion mutant lacking the BAG domain (FLAG-(Δ421-498)-BAG3. Co-immunoprecipitation (IP) analysis with FLAG antibodies was performed using rabbit IgG (rb IgG) as negative control. Left panel shows relative amounts of proteins in cell lysates used for Co-IP. Right panel shows levels of Hsc/Hsp70 and FLAG-tagged proteins in FLAG immunoprecipitates. (**D**) Representative microscope images of HEK cells transfected for 18 h with SOD^{WT}-GFP along with FLAG-tagged BAG3 (FLAG-BAG3) and HA-tagged Hsp70 (HA-Hsp70), as indicated. Scale bar: 10 µm. (**E**) d2HEK cells, which stably express the GFP-based proteasome reporter d2GFP (Gamerdinger et al, 2009), were transfected with HA-tagged ubiquitin (HA-Ubiquitin) and 6 h after transfection treated with the E1 inhibitor PYR-41 (10 µM, 12 h) to block ubiquitination. GFP fusion proteins were then immunoprecipitated and analyzed for HA immunoreactivity. Lower panel shows relative amounts of proteins in total lysates. (**F**) Microscope images of cells transfected as in (Fig 2B) showing altered localization of SOD^{G85R}-GFP. Scale bar: 10 µm.

Fig. S3 (**A**)-(**E**) Immunofluorescence images showing entire cells and single fluorescence channels from analysis presented in Fig. 3A. Arrows in (D) and (E) point to clusters of LC3-positive autophagosomes and LAMP2-positive lysosomes, respectively, which co-localize with mtSOD in the aggresome. Scale bar: 10 µm. (**F**) Representative immunoblot of data displayed in the diagram of Fig. 3F. (**G**) Immunoblot analysis of spinal cord extracts from non-transgenic (nTg), SOD^{WT} (WT) and mtSOD (G93A, G85R, both at endstage) mice. Left panel shows relative levels of indicated proteins in total lysates. Right panel shows the relative levels of these proteins after enrichment of aggregates by differential centrifugation.

Figure S1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Vinblastine - - + +
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B
Dot Blot
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nons
bag3
1
.5
0
**
DMSO
MG132
Ratio: Insoluble/Soluble
C
Soluble
Insoluble
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DMSO
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Ratio: Insoluble/Soluble
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F
BAG3-GFP
Vimentin
Merge
MG132
MG132 + Vinblastine
G
time after MG132 exposure
ctrl
4 h
24 h
24 h + BafA1
48 h
Vector
BAG3
H
BAG3 overexpression
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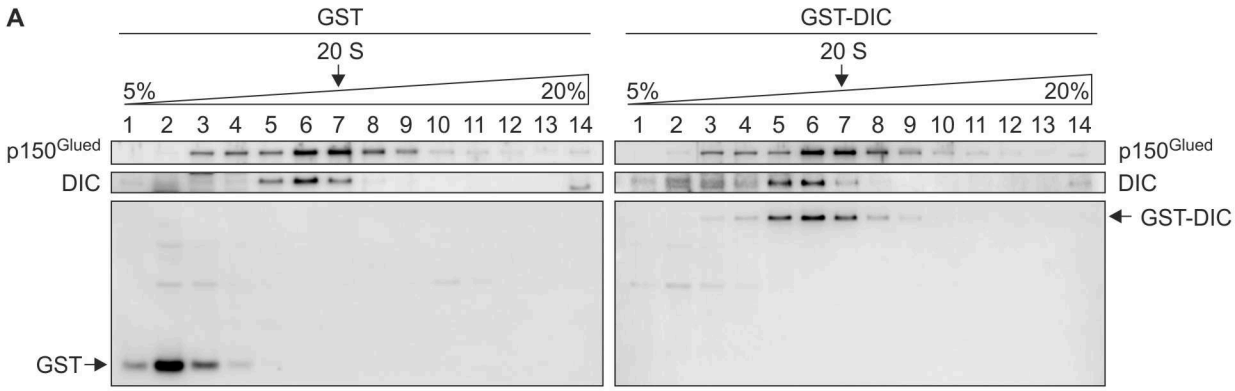
BafA1

Figure S2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20 S
5%
20%
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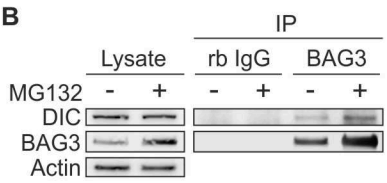
p150Glued
DIC
GST
GST-DIC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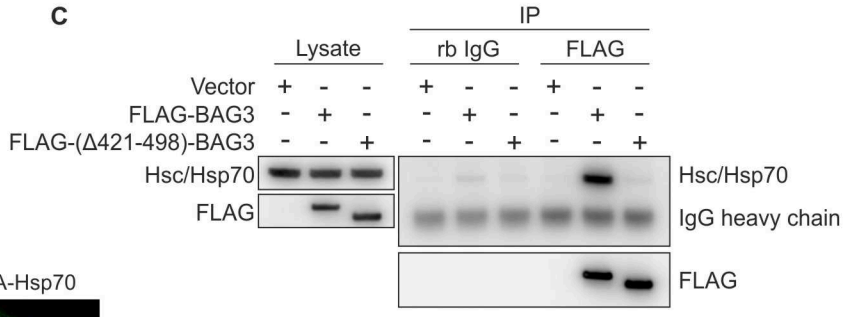
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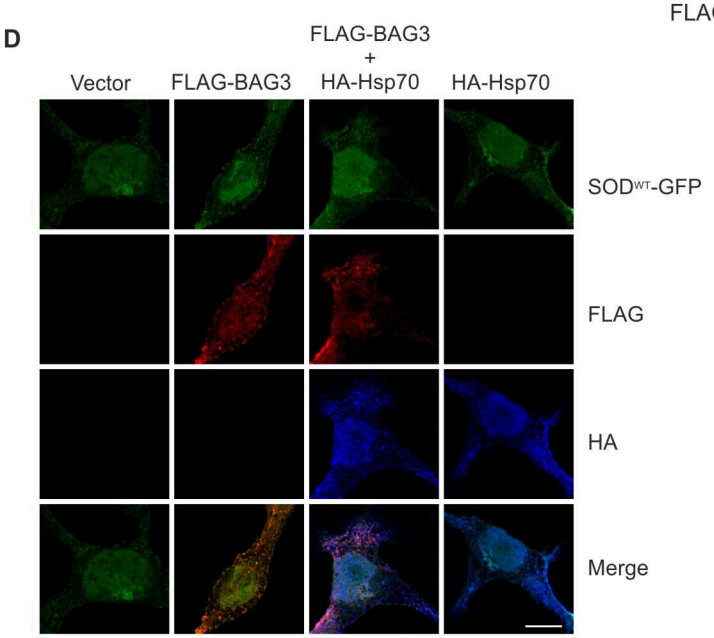
FLAG
IgG heavy chain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FLAG
HA
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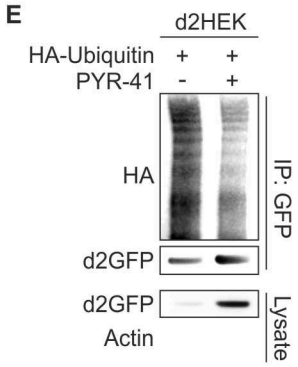
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HA
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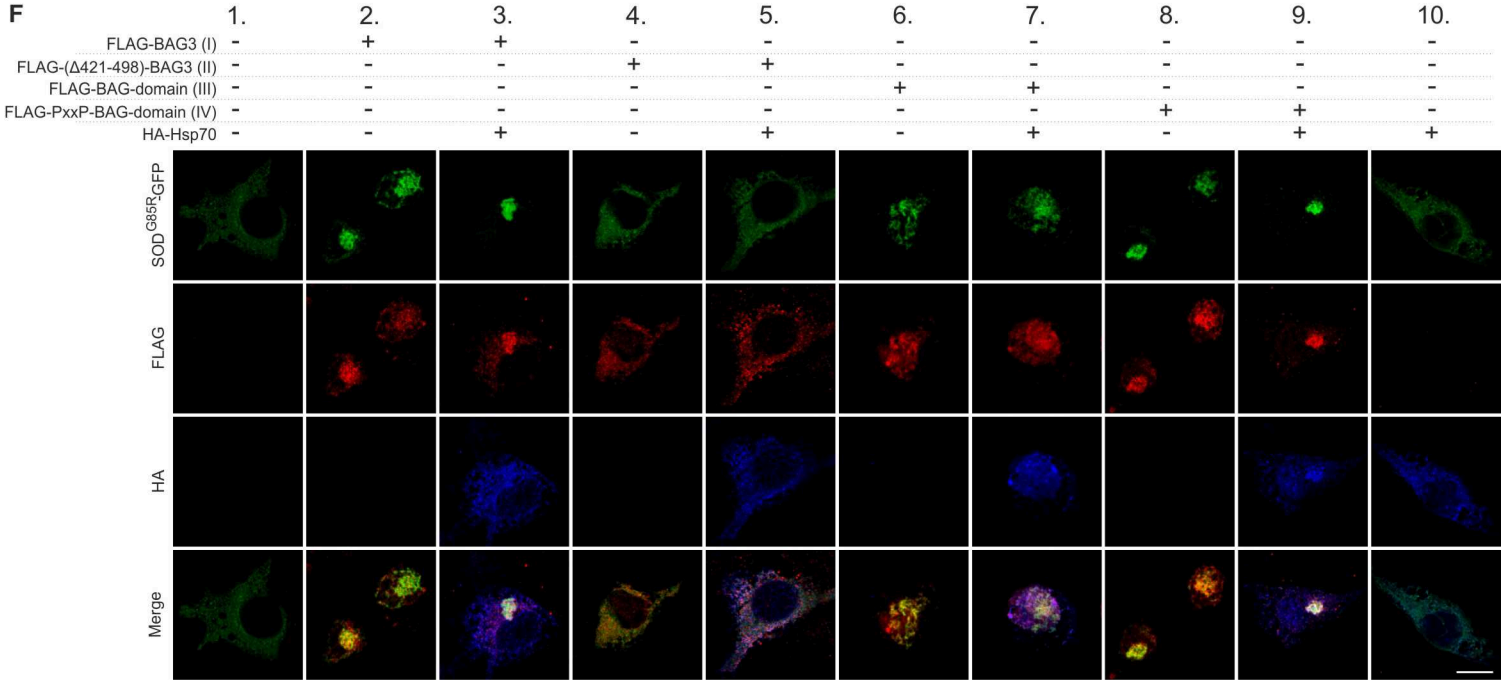
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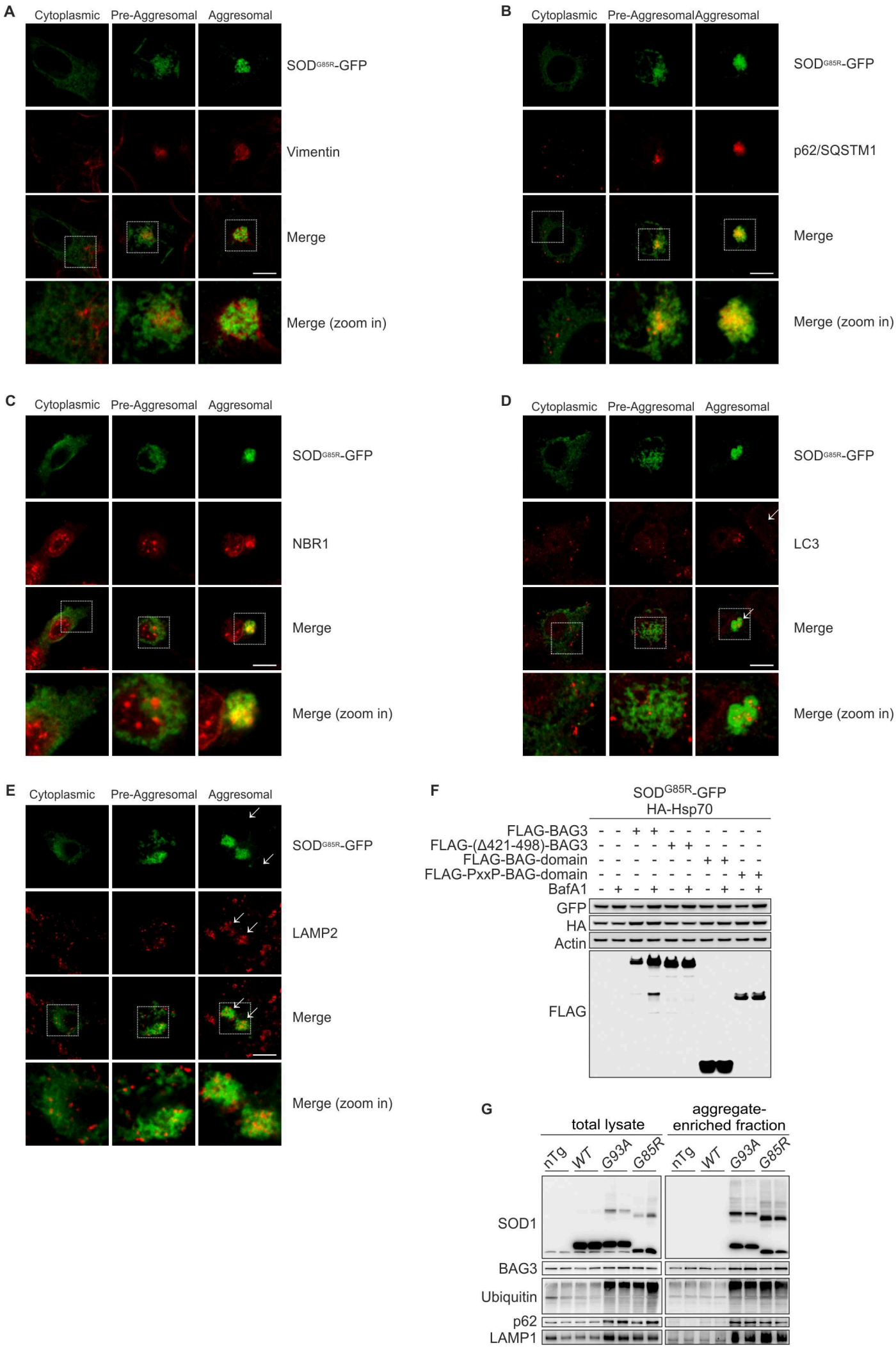
HA-Hsp70
SOD G85R GFP
FLAG
HA
Merge

Figure S3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Aggresomal
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Vimentin
Merge
Merge (zoom in)
B
Cytoplasmic
Pre-Aggresomal
Aggresomal
SOD^G85R-GFP
p62/SQSTM1
Merge
Merge (zoom in)
C
Cytoplasmic
Pre-Aggresomal
Aggresomal
SOD^G85R-GFP
NBR1
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Merge (zoom in)
D
Cytoplasmic
Pre-Aggresomal
Aggresomal
SOD^G85R-GFP
LC3
Merge
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LAMP2
Merge
Merge (zoom in)
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HA-Hsp70
FLAG-BAG3
FLAG-(Δ421-498)-BAG3
FLAG-BAG-domain
FLAG-PxxP-BAG-domain
BafA1
GFP
HA
Actin
FLAG
G
total lysate
aggregate-enriched fraction
nTg
WT
G93A
G85R
SOD1
BAG3
Ubiquitin
p62
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