Supplementary Figure Legends

Figure S1. AMBRA1 interacts with the anti-apoptotic factor BCL-2 in mammalian cells

HEK293 cells were co-transfected with vectors encoding FLAG-BCL-2 and myc-AMBRA1 FL (Full length), myc-β-Galactosidase (βGal) as a negative control or myc-AMBRA1 mutants F1, F2 or F3. Protein extracts were immunoprecipitated using an anti-FLAG antibody. Purified complexes and corresponding total extracts were analyzed by Western Blot (WB) using an anti-BCL-2 and anti-myc antibodies. Asterisks point to the molecular weight proteins corresponding to the original AMBRA1 fragments.

Figure S2. AMBRA1 interacts with the mitochondrial targeted-BCL-2 and this interaction is reduced after autophagy induction

(a) HEK293 cells were co-transfected with vectors encoding myc-AMBRA1 FL and either mito-BCL-2 or ER-BCL-2. Protein extracts were immunoprecipitated using an anti-BCL-2 antibody. Purified complexes and corresponding total extracts were analysed by WB, by using anti-BCL-2 and anti-myc antibodies.

(b) HEK293 cells were co-transfected with vectors encoding myc-AMBRA1 FL and ER-BCL-2 or ER-BCL-2 alone (negative control). Cells were grown either in normal media or in EBSS media for 4 hours. Protein extracts were immunoprecipitated using an anti-myc antibody. Purified complexes and corresponding total extracts were analysed by WB, by using anti-BCL-2 and anti-myc antibodies. Autophagy induction was measured in total extracts by using an anti-p62 antibody.

(c) HEK293 cells were co-transfected with vectors encoding myc-AMBRA1 FL and mito-BCL-2 or mito-BCL-2 alone (negative control). Cells were grown either in normal media or in EBSS media for 4 hours. Protein extracts were immunoprecipitated using an anti-myc

antibody. Purified complexes and corresponding total extracts were analysed by WB, by using anti-BCL-2 and anti-myc antibodies. Autophagy induction was measured in total extracts by using an anti-p62 antibody.

(d) HEK293 cells were transfected with a vector encoding myc-AMBRA1 FL or mito-BCL-2 or ER-BCL-2 alone (negative controls). Cells were grown either in normal media or in EBSS media for 4 hours. Protein extracts were immunoprecipitated using an anti-myc antibody. Purified complexes and corresponding total extracts were analysed by WB, by using anti-BCL-2 and anti-myc antibodies.

(e) HEK293 cells were grown in normal or in EBSS media for 4 hours. Mitochondrial fractions were then isolated by centrifugation and the quality of the fractions controlled by WB by using anti-MnSOD and anti-GAPDH antibodies. Endogenous mitochondrial-AMBRA1 was then precipitated using an anti-AMBRA1 polyclonal antibody in both conditions. Purified complexes were analysed by WB, by using anti-BECLIN 1, anti-BCL-2 and anti-AMBRA1 antibodies. Densitometry analysis was performed in order to estimate BECLIN 1-AMBRA1 binding and BCL-2-AMBRA1 binding.

Figure S3. Only a small pool of ER-BCL-2 can be found associated to AMBRA1

(a) AMBRA1 scarcely co-localizes with ER-BCL2. Confocal analysis of HEK293 co-transfected with vectors coding for ER-BCL-2 and for myc-AMBRA1 grown either in normal media or in EBSS for 4 hours and stained with an anti-myc-AMBRA1 (red) and anti-BCL-2 antibodies (green). Background of the image was reduced in order to distinguish ER-BCL-2 The merge of the two fluorescence signals is shown in the right panels together with a higher magnification image (4X). Scale bar, 6 μm. The yellow arrow (Normal conditions) indicates an example of a cell where co-localization between AMBRA1 and ER-BCL-2 is detectable.

(b) The images show microscopic analysis of HEK293 cells co-transfected with vectors encoding ER-BCL-2 and myc-AMBRA1 FL (Full length), grown in normal media and stained with an anti-myc-AMBRA1 (red) and anti-BCL-2 antibody (green). The merge of the two fluorescence signals is shown in the right panels together with a higher magnification (4X). Scale bar, 6 μm. The yellow arrow points to one of the few cells with apparent co-localization. The blue arrows indicate areas where the green and red signals are clearly distinct. The asterisk indicates the co-localising areas. The white arrow points to a cell, where no co-localization was detected.

(c) Statistical analysis of AMBRA1-mito versus ER-BCL-2 co-localizations. The Pearson correlation coefficient r_p and Spearman correlation coefficient r_s are indicated on the scatter plot. $*P < 0.05$ versus normal conditions.

Figure S4. Endogenous AMBRA1 co-localizes with endogenous BCL-2 and mitotracker staining

(a) Negative controls of the staining performed in Figure 4. Primary and secondary antibodies were used alone.

(b,c) Endogenous AMBRA1 co-localizes with endogenous BCL-2 under normal conditions (b) and this co-localization is reduced after autophagy induction (c). The images show microscopic analysis of HEK293 cells grown either in normal media (b) or in EBSS (c) for 4 hours and stained with an anti-BCL-2 antibody (blu), an anti-myc-AMBRA1 (green), and mitotracker (red). The merge of the two or three fluorescence signals are shown in the bottom panels together with a higher magnification image (4X). Scale bar, 6 μm. White arrows point to co-localisation areas.

Figure S5. Controls of the Immunogold analysis

(a) Immunogold analysis of purified mitochondria of Hela cells or whole HeLa cells stained with an anti-rabbit antibody. The arrow points to an aspecific 15 nm signal. Scale bar, 0.5 μm.

Figure S6. AMBRA1-mitochondria co-localization quantifications and increase of binding between AMBRA1 and BECLIN 1 after autophagy induction in the microsomal fraction.

(a) Pearson and Spearman Statistical analysis on AMBRA1-mitochondria co-localization in normal conditions and in starvation.

(b) Microsomal fractioning in HEK293 cells. Microsomal fractions were isolated from HEK293 cells co-transfected with both AMBRA1 and BECLIN 1, grown either in normal media or in EBSS for 4 hours. The microsomal fraction was controlled using an anti-MnSOD antibody (as a negative control) and using an antibody against the microsomal enzyme Protein Disulphide Isomerase (PDI protein). Microsomal fractions were immunoprecipitated using an anti-FLAG antibody (FLAG-Beclin 1). Purified complexes and corresponding total extracts were analysed by WB using an anti-myc antibody or an anti-BECLIN 1 antibody.

(c) Microsomal fractioning in HEK293 cells. Microsomal fractions were isolated from HEK293 cells grown either in normal media or in EBSS for 4 hours. The microsomal fraction was controlled using an anti-MnSOD antibody (as a negative control) and using an antibody against the microsomal enzyme Protein Disulphide Isomerase (PDI protein). Microsomal fractions were immunoprecipitated using an anti-BECLIN 1 antibody. Purified complexes and corresponding total extracts were analysed by WB using an anti-BCL-2 antibody or an anti-BECLIN 1 antibody.

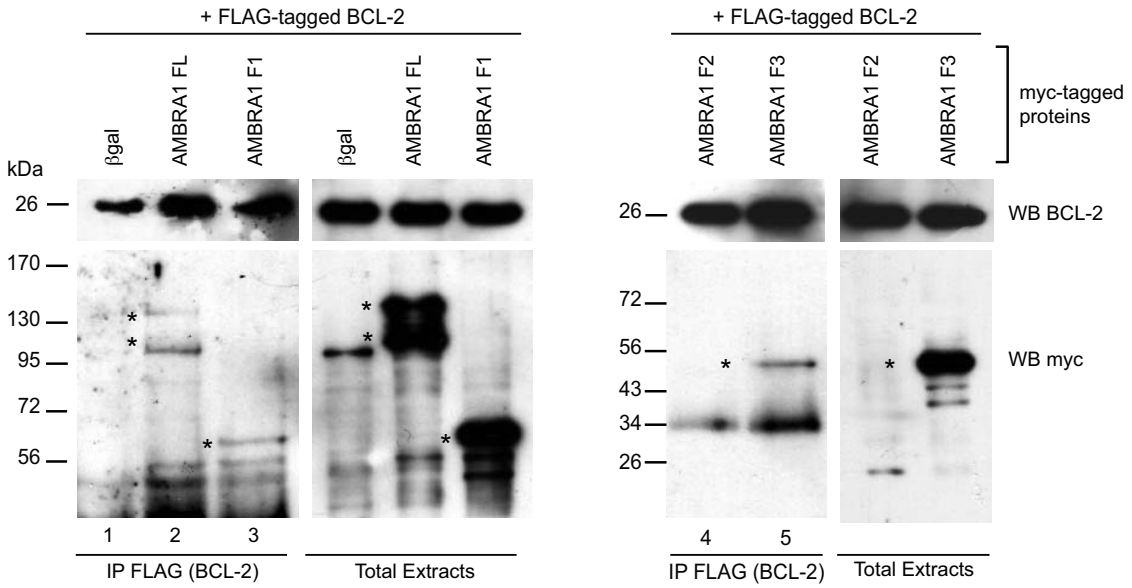
Figure S7. mito-or ER-BCL-2 can compete with AMBRA1 to bind BECLIN 1

BECLIN 1 and AMBRA1 interaction decreases when mito- or ER-BCL-2 are overexpressed. HEK293 cells were transfected both with FLAG-BECLIN 1 and myc-AMBRA1 and increasing concentrations of *mito-or ER-BCL-2* cDNAs were added. Protein extracts were immunoprecipitated using an anti-FLAG antibody. Purified complexes and corresponding total extracts were analysed by Western Blot (WB), by using anti-BECLIN 1, anti-BCL-2 and anti-AMBRA1 antibodies. BECLIN 1-AMBRA1 binding in the presence or the absence of BCL-2 was quantified by densitometric analysis using the ImageQuant software

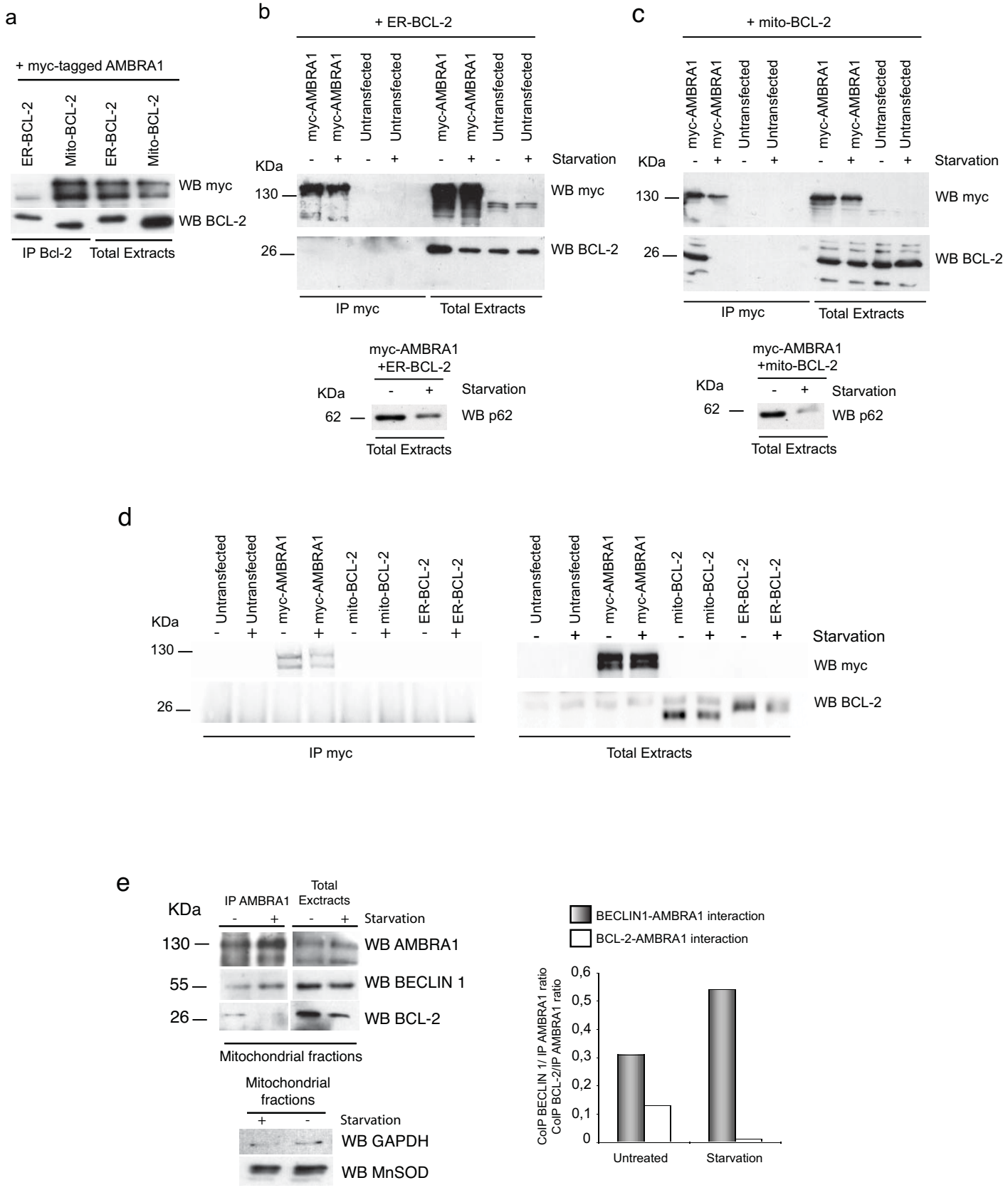
Figure S8. A model of AMBRA1-mitochondrial complex dynamics in normal and autophagy conditions

Under normal conditions, a pool of AMBRA1 is docked by BCL-2 at the mitochondria; After autophagy induction, this AMBRA1 pool competes with Mito-BCL-2 to bind a fraction of mitochondrial BECLIN 1 to participate to the BECLIN 1-dependent autophagic program. In this model we can not exclude the omegasome model: Since a part of AMBRA1-mitochondria co-localization is reduced after autophagy induction (See Figure SD6) we can hypothezise that a pool of mito-AMBRA1 go to the ER in order to bind the resident ER-BECLIN 1 and also enhance the autophagic process.

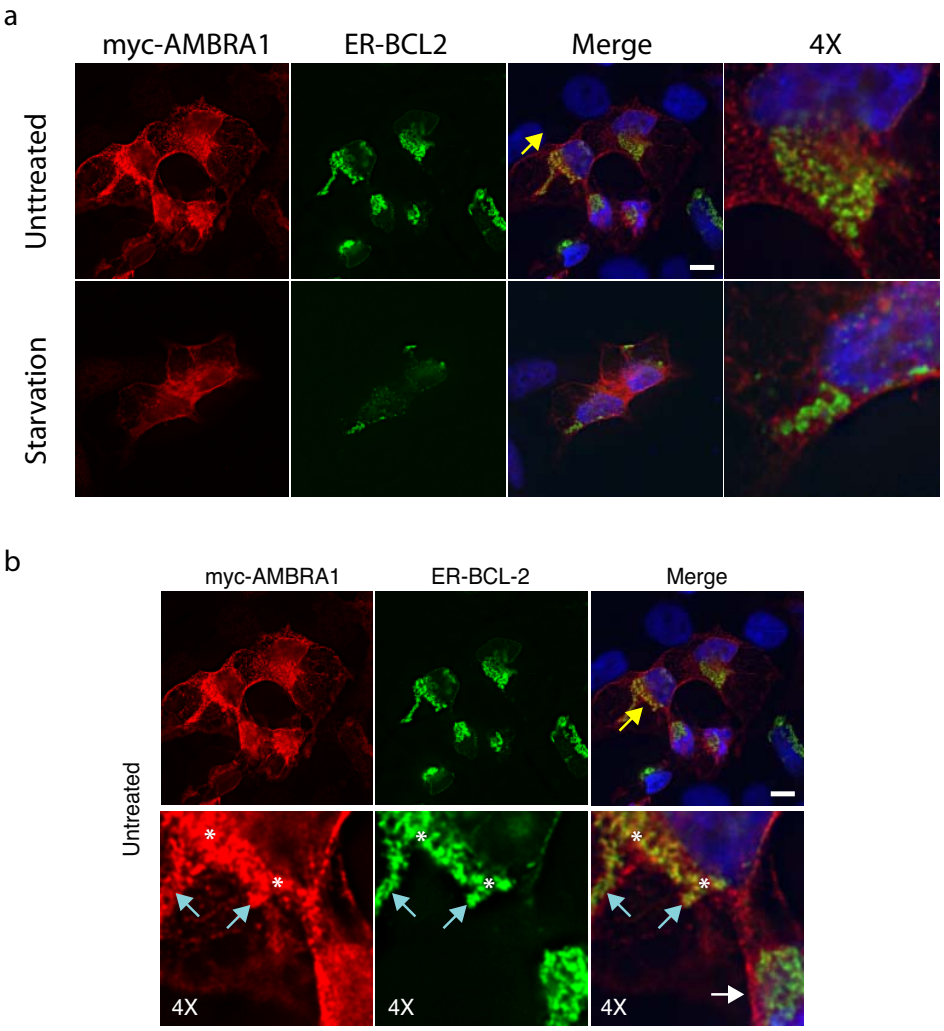
Supplementary Figure S1

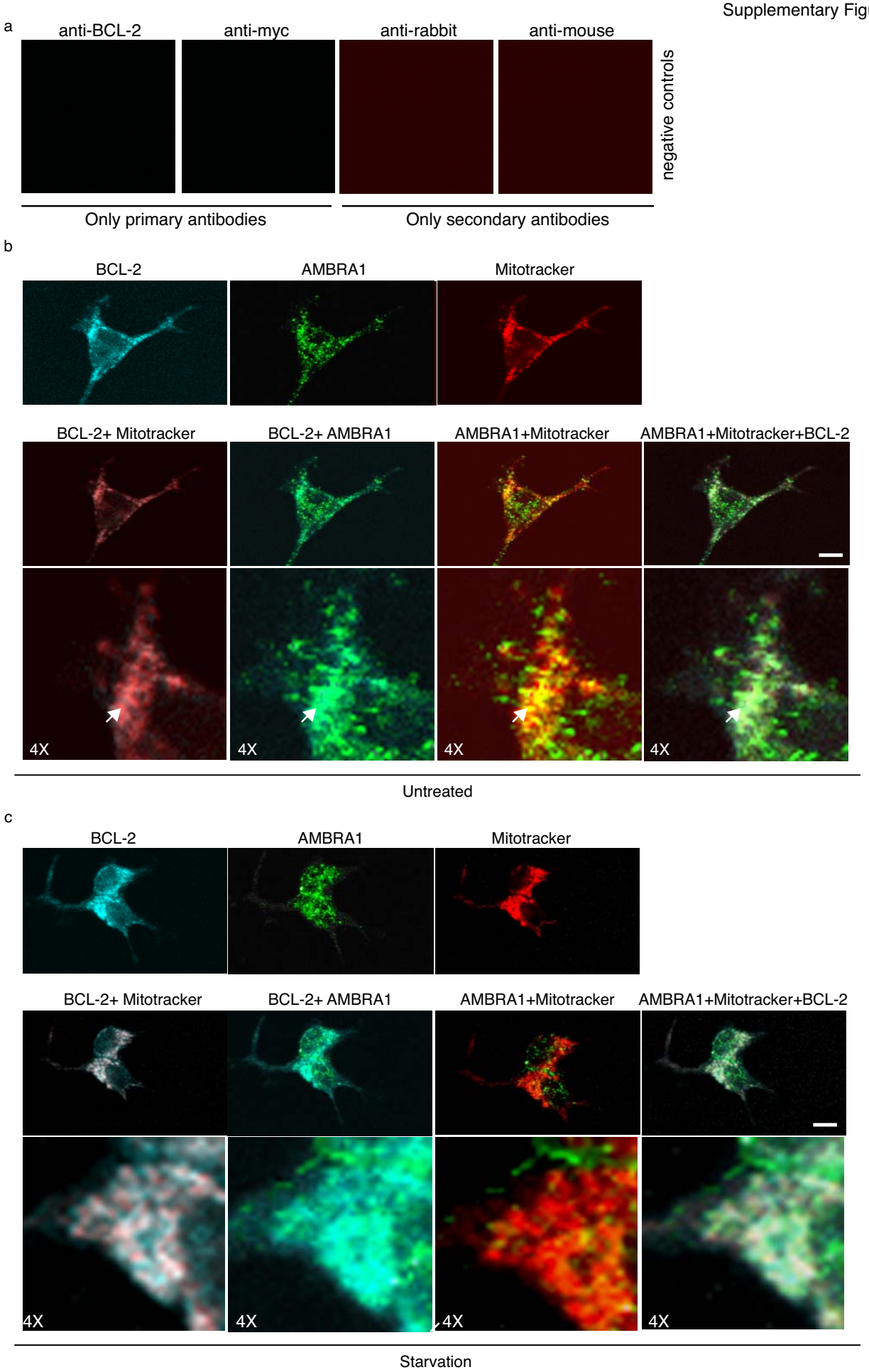


Supplementary Figure S2



Supplementary Figure S3

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anti-BCL-2
anti-myc
anti-rabbit
anti-mouse
negative controls
Only primary antibodies
Only secondary antibodies
b
BCL-2
AMBRA1
Mitotracker
BCL-2+ Mitotracker
BCL-2+ AMBRA1
AMBRA1+Mitotracker
AMBRA1+Mitotracker+BCL-2
4X
Untreated
c
BCL-2
AMBRA1
Mitotracker
BCL-2+ Mitotracker
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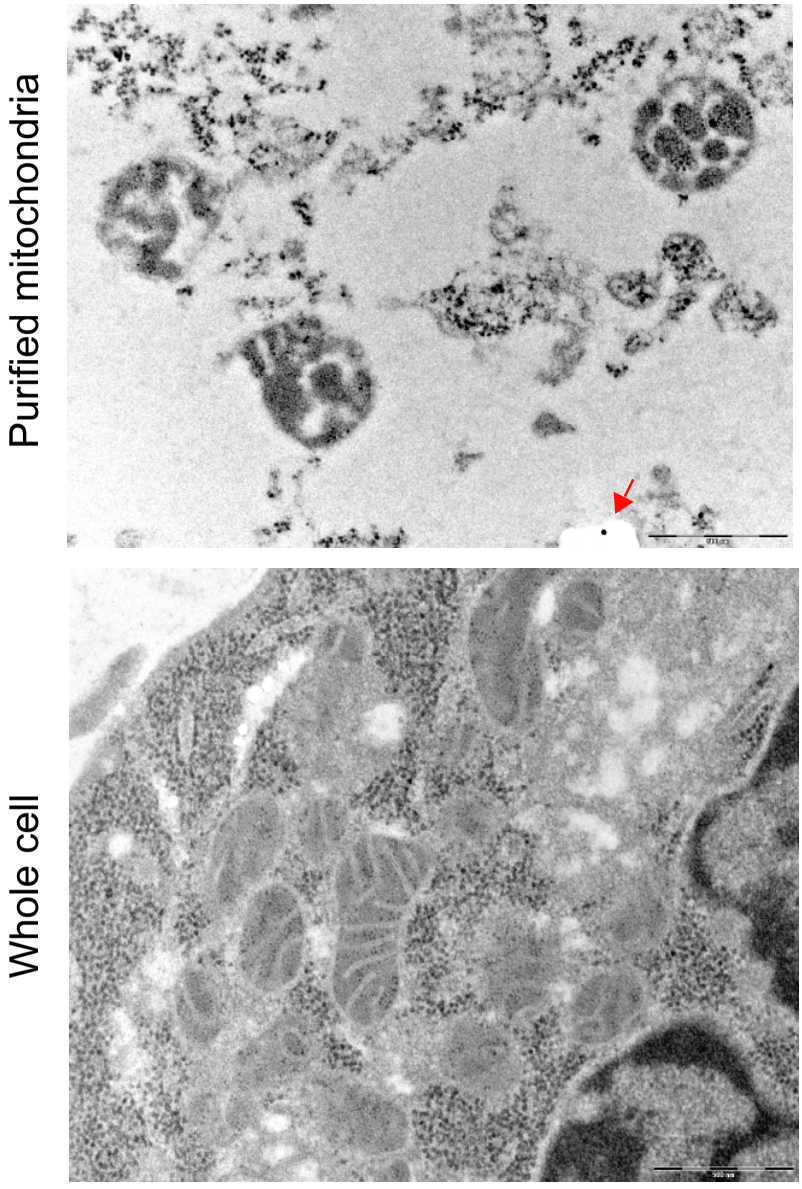
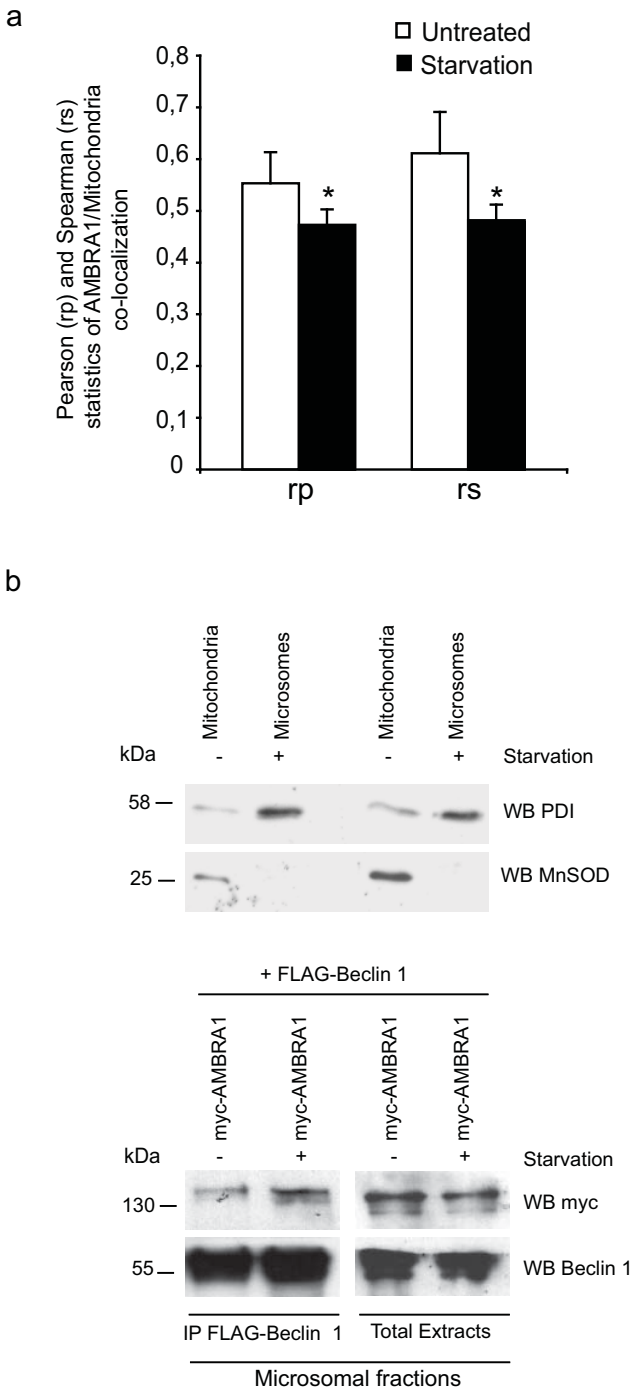
AMBRA1+Mitotracker
AMBRA1+Mitotracker+BCL-2
4X
Starvation

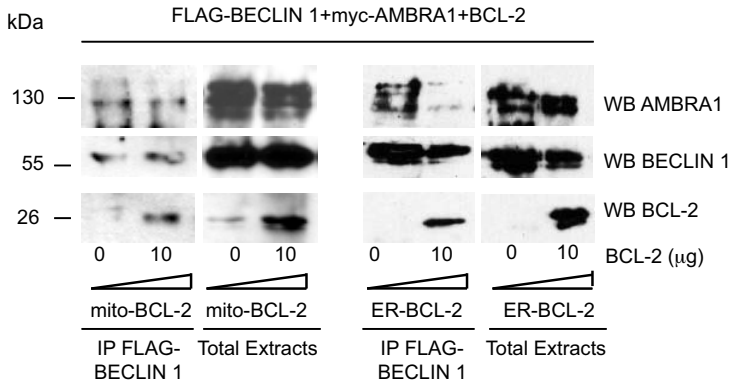
Supplementary Figure S5

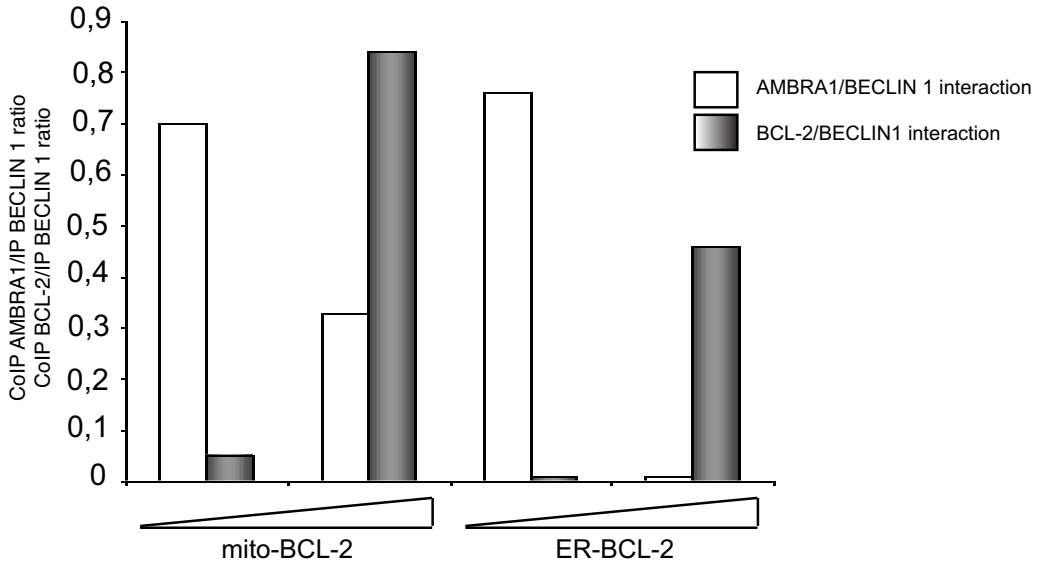


Supplementary Figure S6



Supplementary Figure S7





Supplementary Figure S8