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Supplemental Material to:

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The actin cytoskeleton participates in the early events of autophagosome formation upon starvation induced autophagy

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Figure S1

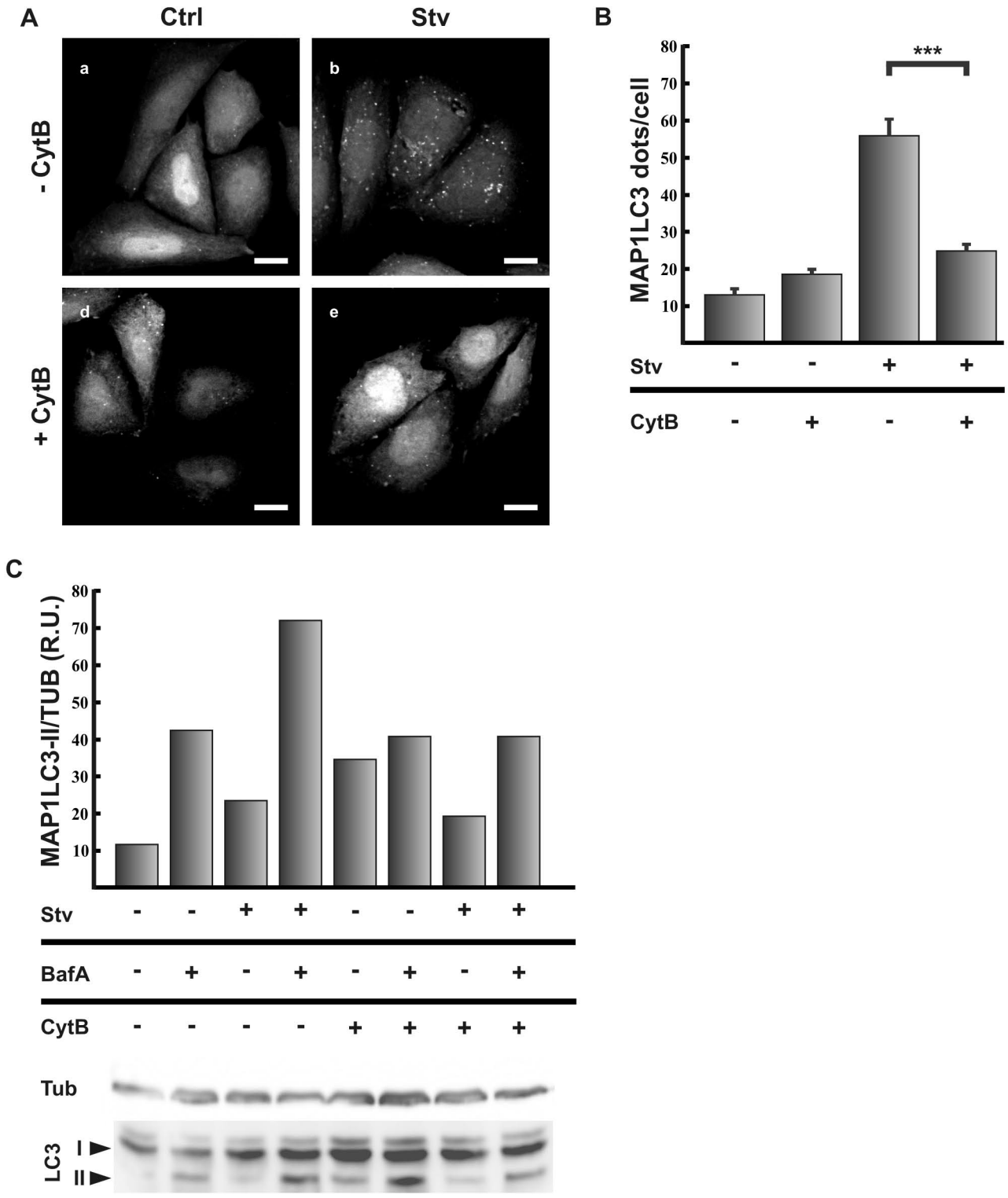
A
Ctrl
Stv
- CytB
+ CytB
a
b
d
e
B
MAP1LC3 dots/cell
80
70
60
50
40
30
20
10
***
Stv - - + +
CytB - + - +
C
MAP1LC3-II/TUB (R.U.)
80
70
60
50
40
30
20
10
Stv - - + + - - + +
BafA - + - + - + - +
CytB - - - - + + + +
Tub
LC3
I
II

Figure S2

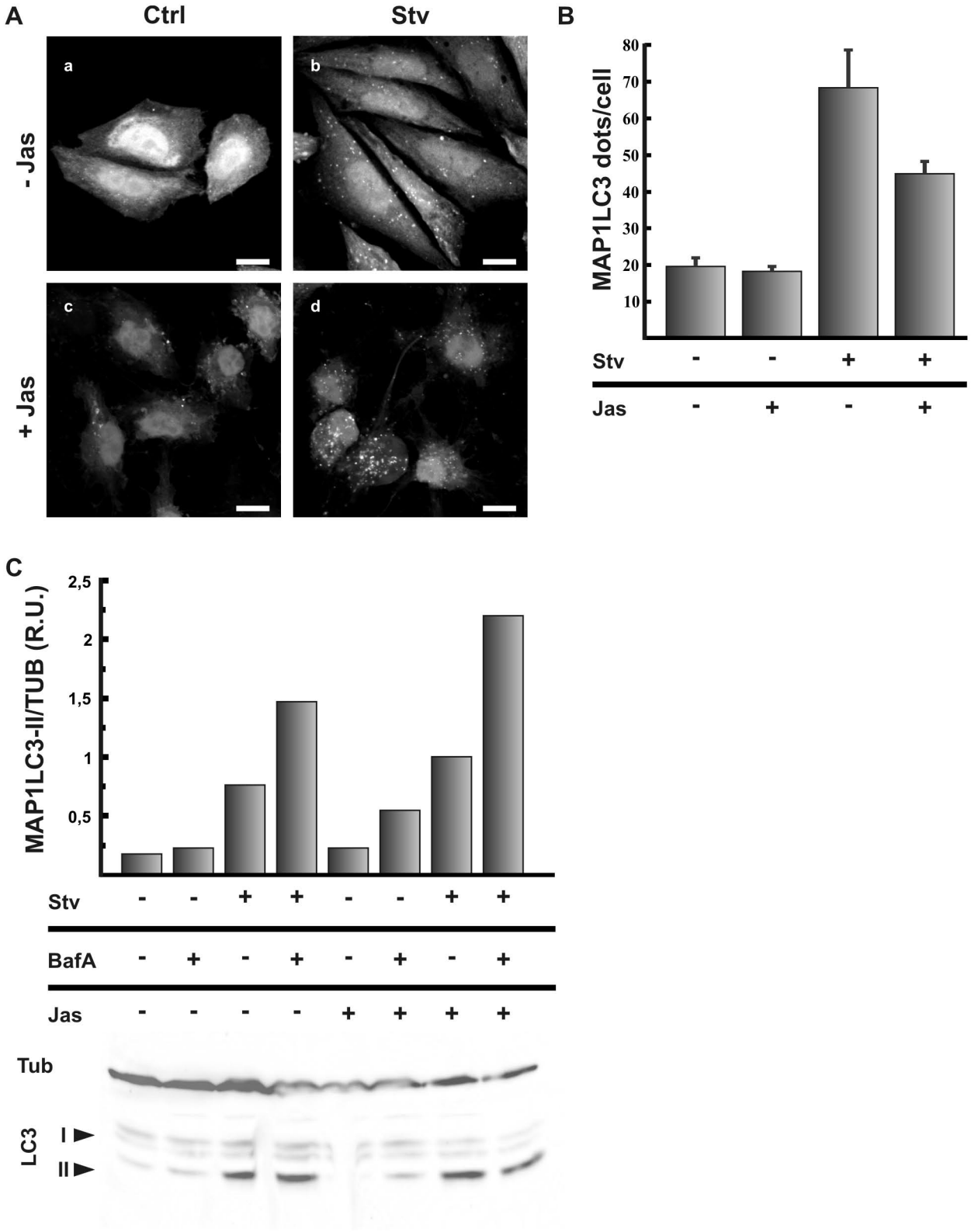
A
Ctrl
Stv
- Jas
+ Jas
a
b
c
d
B
MAP1LC3 dots/cell
80
70
60
50
40
30
20
10
Stv - - + +
Jas - + - +
C
MAP1LC3-II/TUB (R.U.)
2,5
2
1,5
1
0,5
Stv - - + + - - + +
BafA - + - + - + - +
Jas - - - - + + + +
Tub
LC3
I
II

Figure S3

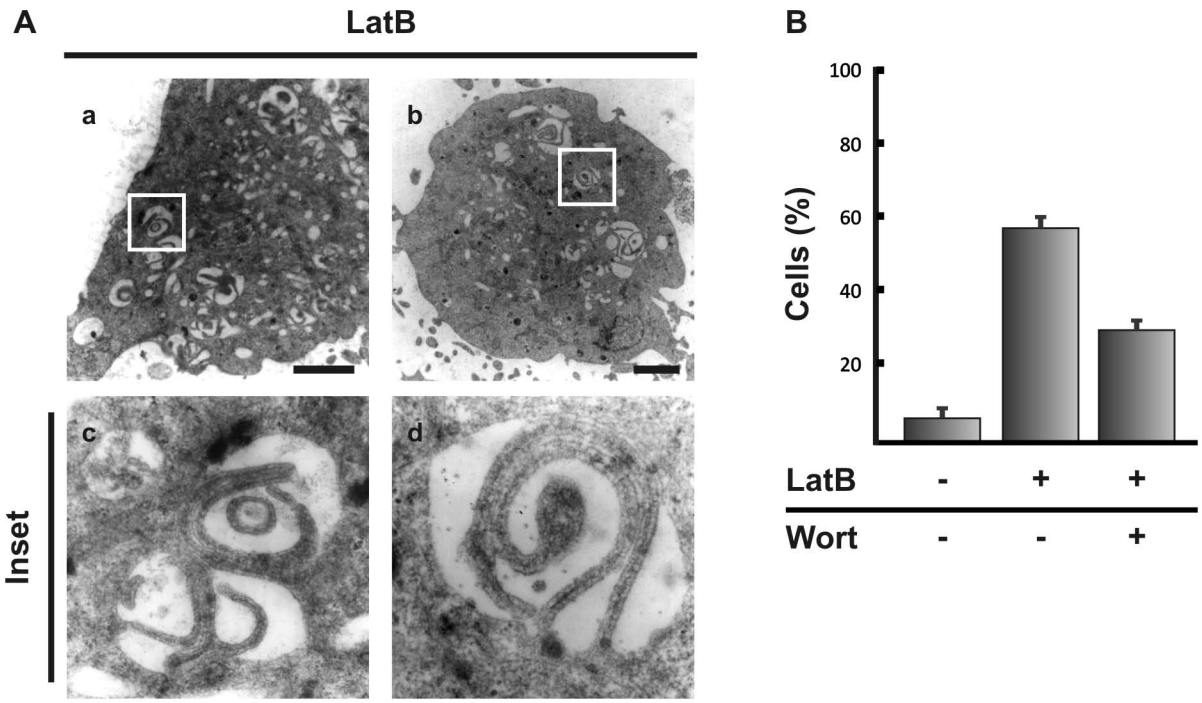

Figure S4

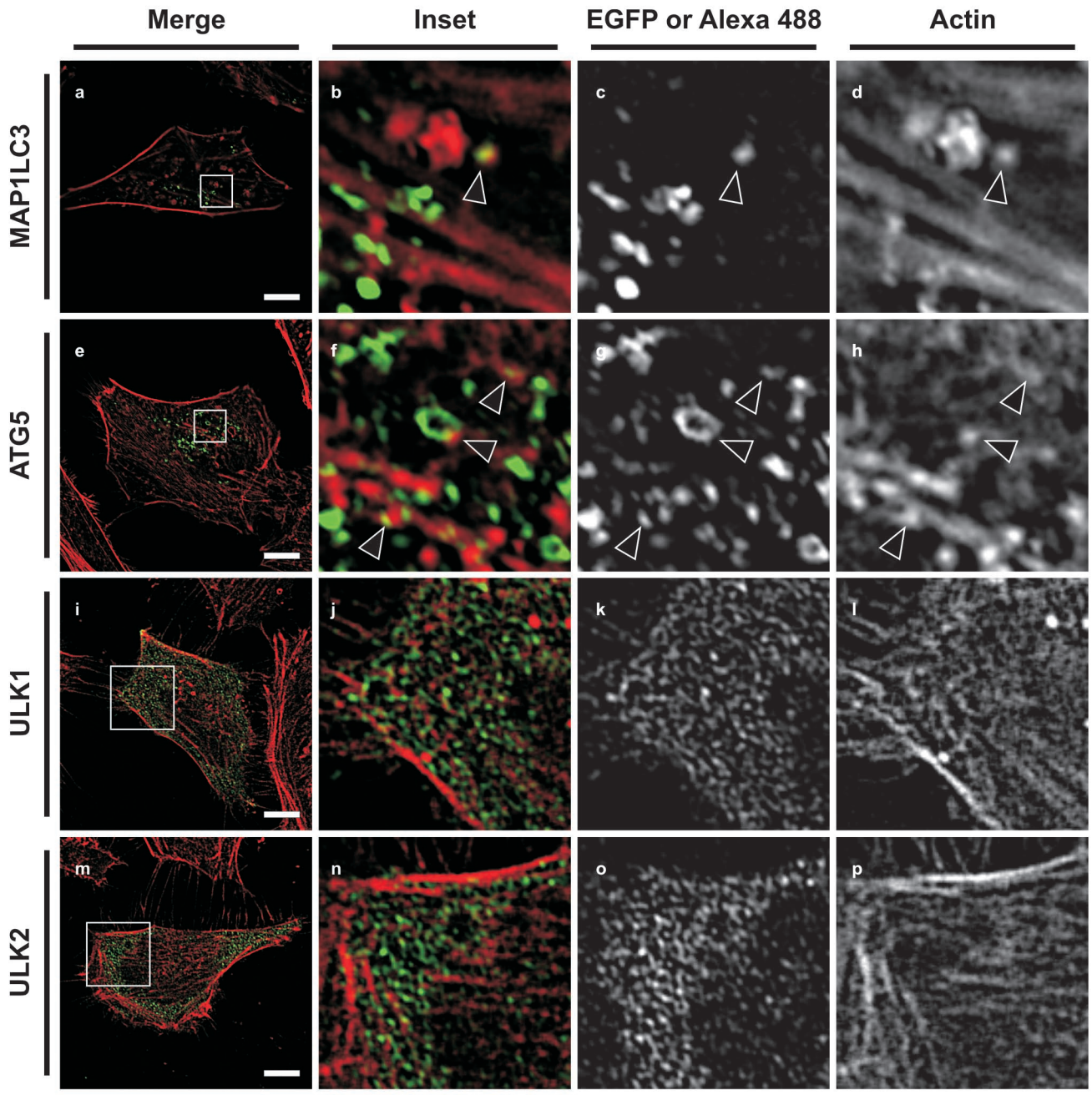
Merge
Inset
EGFP or Alexa 488
Actin
MAP1LC3
ATG5
ULK1
ULK2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure S5

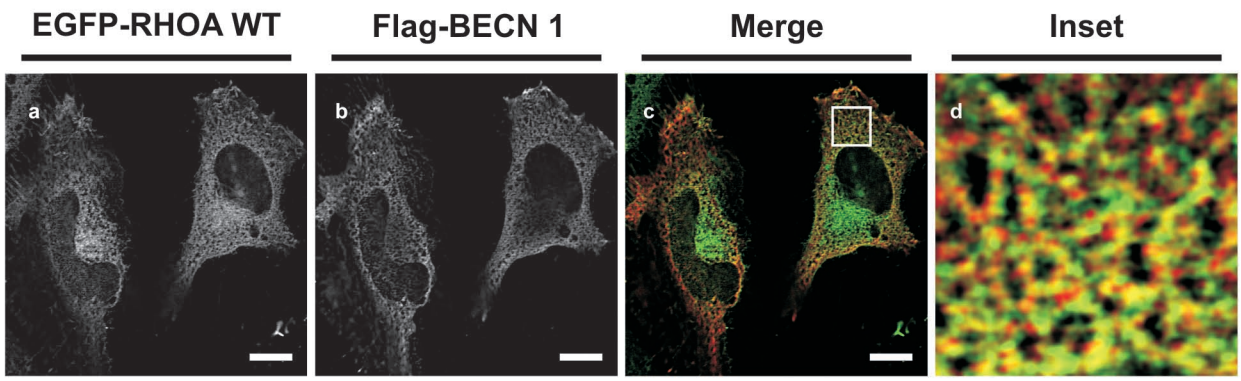

Figure S1. Actin filaments disruption prevented the autophagic response upon starvation. (A) CHO cells stable expressing EGFP-MAP1LC3 were incubated for 2 h at 37ºC in full-nutrient medium (Ctrl) (a and d) or starvation medium (Stv) (b and e); in the presence (d and e) or absence (a and b) of the actin depolymerizing agent Cytochalasin B (CytB) (2.5 μM). Subsequently, cells were fixed in 3% paraformaldehyde and processed for immunofluorescence. (B) The EGFP-MAP1LC3 dots were quantified from max intensity projection of a confocal z-stack and the mean+S.E.M. of the number of dots per cell is shown. The data evaluated correspond to three independent experiments. (C) HeLa cells were incubated in starvation medium in the presence or absence of 2.5 μM Cytochalasin B (CytB), with or without 100 nM bafilomycin A_1 (BafA) for 2 h at 37ºC. Afterwards, cells were lysed in RIPA buffer and the samples were subjected to western blot analysis using a rabbit anti-MAP1LC3 and a mouse anti-TUB antibodies and the corresponding HRP-labeled secondary antibody, and subsequently developed with an enhanced chemiluminescence detection kit. The bands intensity was quantified with ImageJ software (gel analyzer plugin), and the MAP1LC3-II/TUB was calculated. Data shown is representative of two independent experiments. Scale bars shown in (A) represent 10 μm.

Figure S2. Stabilization of actin cytoskeleton does not affect autophagosome formation. (A) CHO cells stable expressing EGFP-MAP1LC3 were incubated for 2 h at 37ºC in full-nutrient medium (Ctrl) (a and c) or starvation medium (Stv) (b and d), in the presence (c and d) or absence (a and b) of the actin stabilizing agent Jasplakinolide (Jas) (1 μM). Subsequently, cells were fixed in 3% paraformaldehyde and processed for immunofluorescence. (B) The EGFP-MAP1LC3 dots were quantified from max intensity projection of a confocal z-stack and the mean+S.E.M. of the number of dots per cell is shown. (C) HeLa cells were incubated in control or starvation medium in the presence or absence of Jasplakinolide (Jas) (1 μM), and/or 100 nM bafilomycin A_1 (BafA) for 2 h at 37ºC. Afterwards, cells were lysed in RIPA buffer and the samples were subjected to western blot analysis using a rabbit anti-MAP1LC3 and mouse anti-TUB antibodies and the corresponding HRP-labeled secondary antibody, and subsequently developed with an enhanced chemiluminescence detection kit. The bands intensity was quantified with ImageJ software (gel analyzer plugin), and the MAP1LC3-II/TUB ratio was calculated. Data shown is representative of two independent experiments. Scale bars depicted in (A) represent 10 μm.

Figure S3. Latrunculin B treatment generates an accumulation of curved membranous structures. (A) HeLa cells were incubated for 2 h at 37ºC in starvation medium in the presence or absence of 10 μM Latrunculin B (LatB), with or without 0.2 μM of wortmannin (Wort). Afterwards, cells were fixed, processed and analyzed by electron microscopy using conventional techniques. In the micrographs and in the insets membranous whirled structures that may represent aberrant isolation membranes or phagophores are visualized. (B) Quantification of the percentage of cells with this type of structures. Scale bars shown in (A) represent 5 μm.

Figure S4. Actin filaments do not colocalize with certain autophagic markers. HeLa cells overexpressing GFP-MAP1LC3 (a-d), EGFP-ATG5 (e-h), myc-ULK1 (i-l), or myc-ULK2 (m-p), were incubated in starvation medium for 2 h at 37ºC. Subsequently, cells were fixed in 3% paraformaldehyde and processed for immunofluorescence and actin filaments were stained using Phalloidin-Rhodamine. To stain myc-ULK1/2, a mouse anti-myc antibody and an anti-mouse Alexa Fluor 488 secondary antibody was used. Scale bars represent 10 μm. Arrowheads indicate colocalization sites.

Figure S5. RHOA colocalizes with BECN1. HeLa cells overpressing Flag-BECN1 1 (a-d), were incubated in starvation medium for 2 h at 37ºC. Subsequently, cells were fixed in 3% paraformaldehyde and processed for immunofluorescence and actin filaments were stained using Phalloidin-Rhodamine. To stain FLAG-BECN1 1, a mouse anti-flag antibody and an anti-mouse Alexa Fluor 488 secondary antibody was used. Scale bars represent 10 μm.