SUPPLEMENTARY DATA

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Mutagenesis of EGFP-MAP1LC3β— A plasmid encoding human MAP1LC3β fused to the COOH-terminus of green fluorescent protein was obtained from Addgene (1) and used to introduce silent mutations within the MAP1LC3β sequence using the oligonucleotide primers 5'-CATCCAACCAAAATCCCGGT**A**ATAATAGA**G**CGATACAAGGGTGAGA-3' (sense) and 5'-TCTCACCCTTGTATCG**C**TCTATTAT**T**ACCGGGATTTTGGTTGGATG-3' (antisense), and the Quikchange™ mutagenesis kit (Stratagene). The resulting plasmid was sequenced to verify the specific mutations and the absence of PCR errors.

Rescue of TLR3-mediated Type I Interferon Signaling by Mutant EGFP-MAP1LC3β— To restore type I interferon signaling activity in WT TLR3-HA expressing 293 cells following LC3β siRNA-A-mediated knockdown of MAP1LC3β expression, cells were transfected with mutant EGFP-MAP1LC3β encoding plasmid as follows. WT cells treated with wild type LC3β siRNA-A duplex and growing on a 60-mm culture dish were harvested and one fourth of the cells was pelleted and suspended in a transfection mixture consisting of 0.5 μg of mutant EGFP-MAP1LC3β plasmid, 2.5 μg of pISRE-LUC reporter, 100 pmoles of wild type LC3β siRNA-A duplex and 10 μl of Lipofectamine 2000™ in 350 μl of Opti®-MEM plus 2 ml of DMEM without antibiotics. One hundred microliter samples of the suspended cells were then plated in 8 wells of a 96-well plate and grown overnight at 37°C. Cells were stimulated with 25 μg/ml of poly I:C and the luciferase activity was measured with 100 μl of Bright-Glo™ reagent. Luciferase activity was normalized to the expression of TLR3-HA and expressed relative to the activity detected in the unstimulated WT-TLR3 expressing cells transfected with GFP-MAP1LC3β encoding plasmid alone. Expression of GFP-MAP1LC3β fusion proteins was monitored by Western blotting with a monoclonal antibody to GFP (Santa Cruz Biotechnology) or polyclonal anti-MAP1LC3β antibodies (Novus Biologicals).

Mutagenesis of Beclin 1— The plasmid pcDNA3-Beclin 1 encoding the human Beclin 1 protein was purchased from Addgene (2) and used as a template to introduce silent point mutations corresponding to those present in the Beclin 1 siRNA-C duplex (*see* Table 1) sequence using the oligonucleotides 5'CTTAGAGCAAATGAATGAGGA**C**GA**T**AGTGAACA**A**TTACAGATGGAGCTAAAGGAG-3' (sense) and 5'-CTCCTTTAGCTCCATCTGTAA**T**TGTTCACT**A**TC**G**TCCTCATTCATTTGCTCTAAG-3' (antisense), and the Quikchange™ mutagenesis kit. The resulting construct was fully sequenced to verify the presence of the desired mutations and the absence of PCR errors.

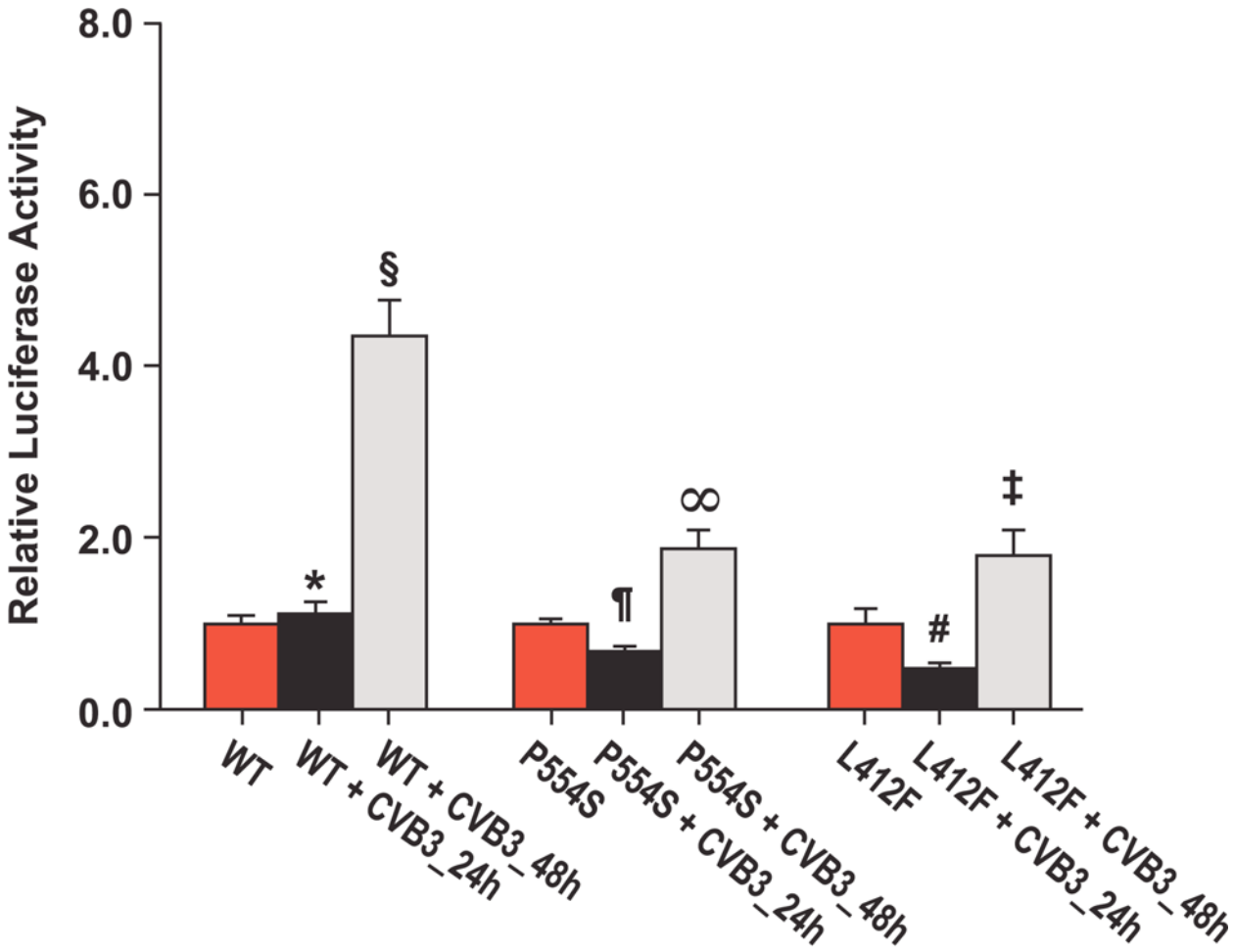
Rescue of TLR3-mediated Type I Interferon Signaling by Wild Type Beclin 1— To restore type I interferon signaling in WT TLR3-HA cells subjected to two rounds of Beclin 1 RNAi, cells were co-transfected a third time with siRNA-C duplex and either WT Beclin 1 encoding plasmid, or the mutant pcDNA3-Beclin 1 construct as follows. The harvested cells were suspended in 14 ml of media, dispensed into 4-ml aliquots and centrifuged. The cell pellets were then resuspended in transfection mixture consisting of either 50 pmoles of siRNA-C plus 2.5 μg of pISRE-LUC reporter, or 50 pmoles of siRNA-C, 2.5 μg of reporter plus 0.5 μg of either WT or mutant pcDNA3-Beclin 1 plasmid in 330-μl of Opti™-MEM containing 13.2 μl of Lipofectamine 2000™. 1.9-ml of DMEM without antibiotics was added to each cell suspension, and 100-μl aliquots were then plated in 16 wells of a 96-well plate and grown overnight at 37°C. Cells in eight of the wells were stimulated for 24 h in 100 μl of DMEM plus antibiotics containing 25 μg/ml poly I:C and the luciferase activity was measured as described above. Luciferase activity was normalized to TLR3-HA expression and expressed relative to the activity detected in unstimulated WT TLR3 cells transfected with reporter alone. Expression of Beclin 1 was monitored by Western blotting

with a monoclonal antibody (Santa Cruz Biotechnology) in the whole cell extracts from ~0.6-ml samples of the cell suspensions described above grown in parallel in a 24-well plate.

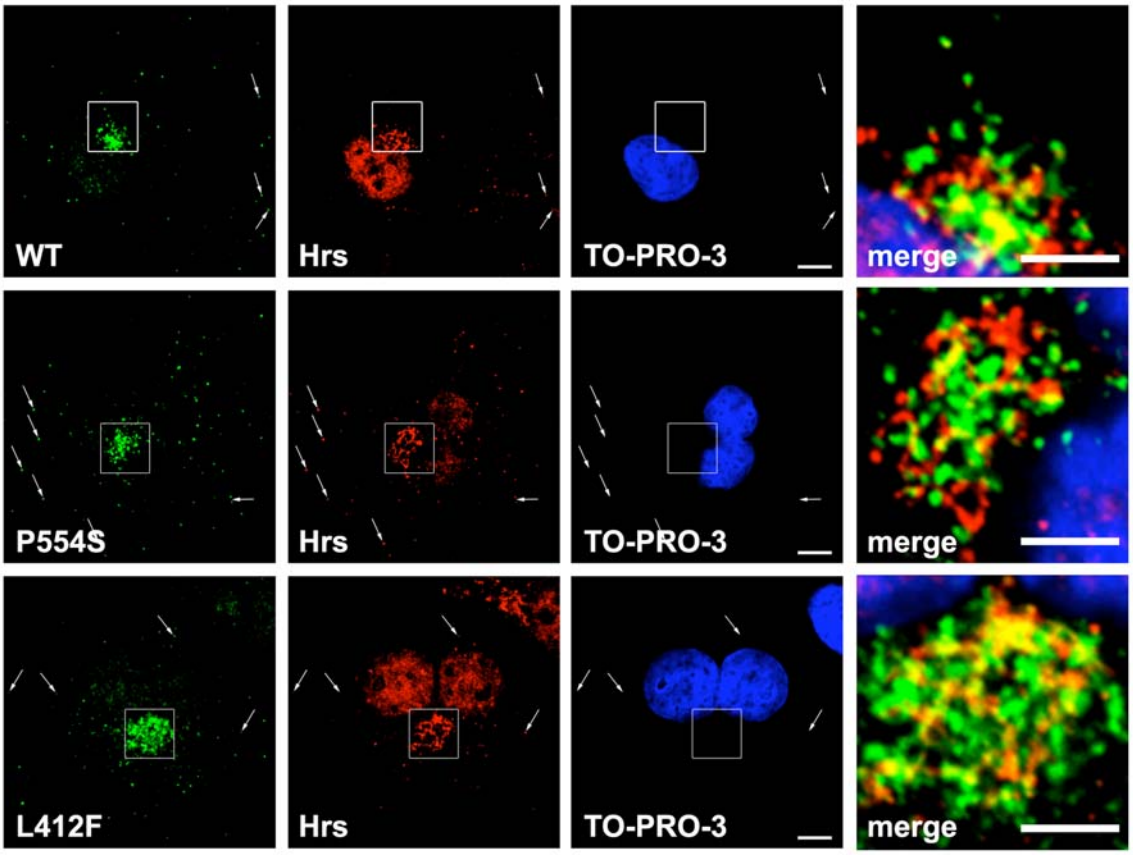
SUPPLEMENTARY REFERENCES

1. Jackson, W. T., Giddings, T. H., Jr., Taylor, M. P., Mulinyawe, S., Rabinovitch, M., Kopito, R. R., and Kirkegaard, K. (2005) *PLoS Biol.* **3**, e156
2. Shihibata, M., Lu, T., Furuya, T., Degterev, A., Mizushima, N., Yoshimori, T., MacDonald, M., Yankner, B., and Yuan, J. (2006) *J. Biol. Chem.* **281**, 14474-14485

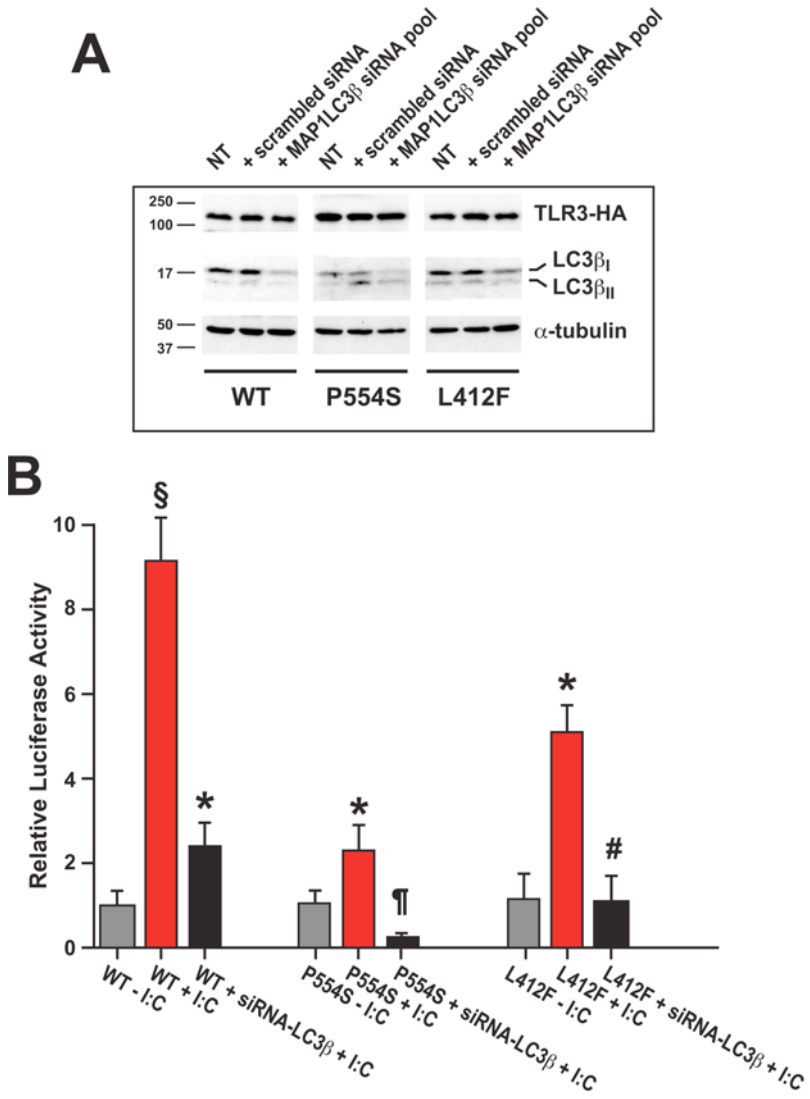
SUPPLEMENTARY FIGURES



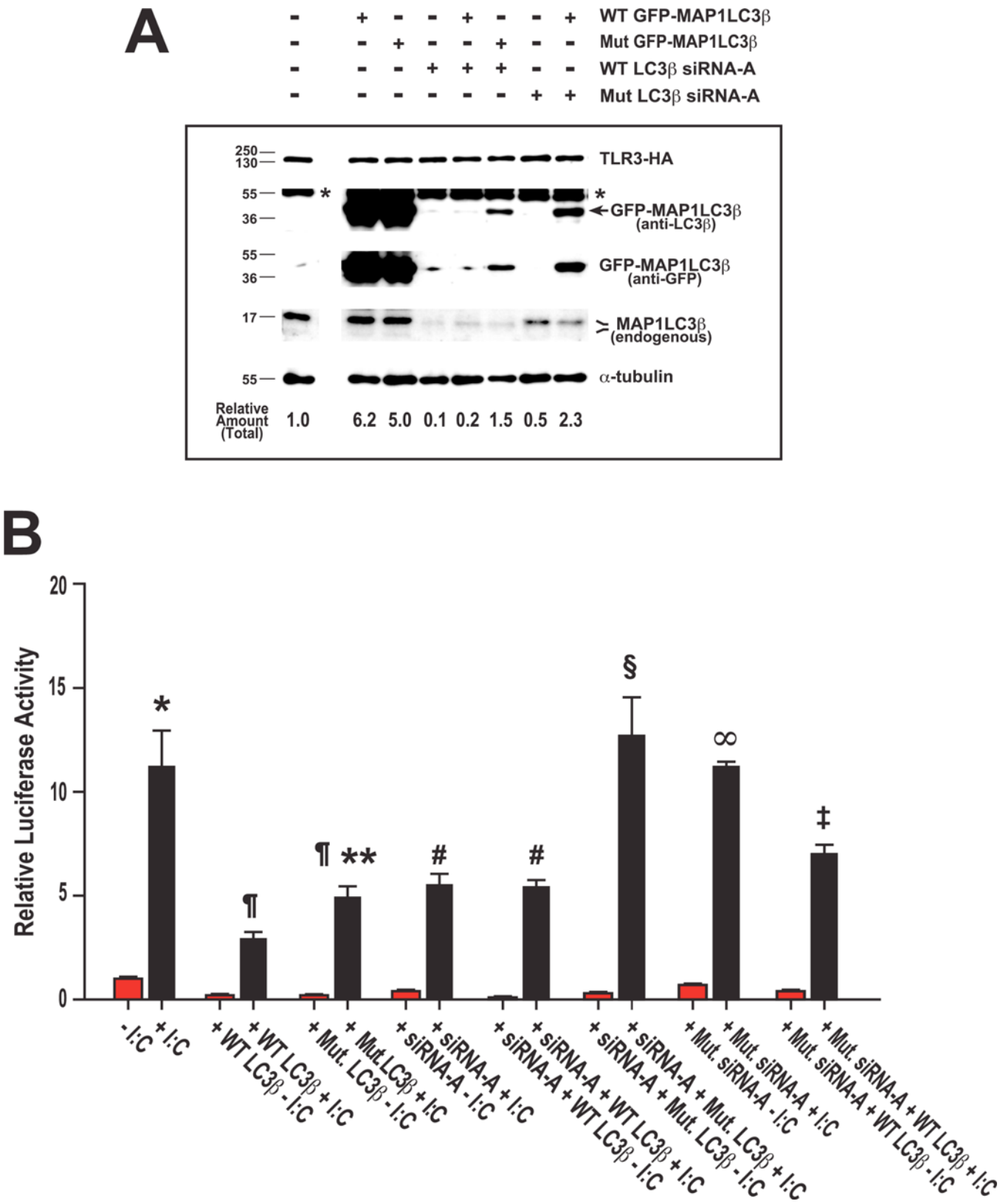
Supplementary Figure 1: Analysis of the effect of TLR3 variants on type I interferon signaling following enterovirus infection (MOI = 0.01). Stable cell lines expressing WT, Ser554 (P554S) or Phe412 (L412F) TLR3-HA were transfected with pISRE-LUC and then stimulated infected with Coxsackievirus B3. Luciferase activity was measured at 0 (*red bars*), 24 (*black bars*) and 48 (*grey bars*) hours post-infection, normalized to exogenous TLR3 expression and expressed relative to uninfected cells. The data shown are the *means* ± *SEM* of eight independent measurements. * P=NS *vs.* unstimulated WT. § P<0.002 *vs*. uninfected WT and all other groups. ¶ P=NS *vs*. uninfected cells in all groups. ∞ P<0.002 *versus* uninfected Ser554 or infected Ser554 for 24 hours. # P=NS *vs*. uninfected or 24-hour infected cells in all groups. ‡ P<0.002 *versus* uninfected cells in all groups or Phe 412 cells infected for 24 hours; P=NS *vs*. 48-hour infected Ser554 cells or WT cells infected for 24 hours.



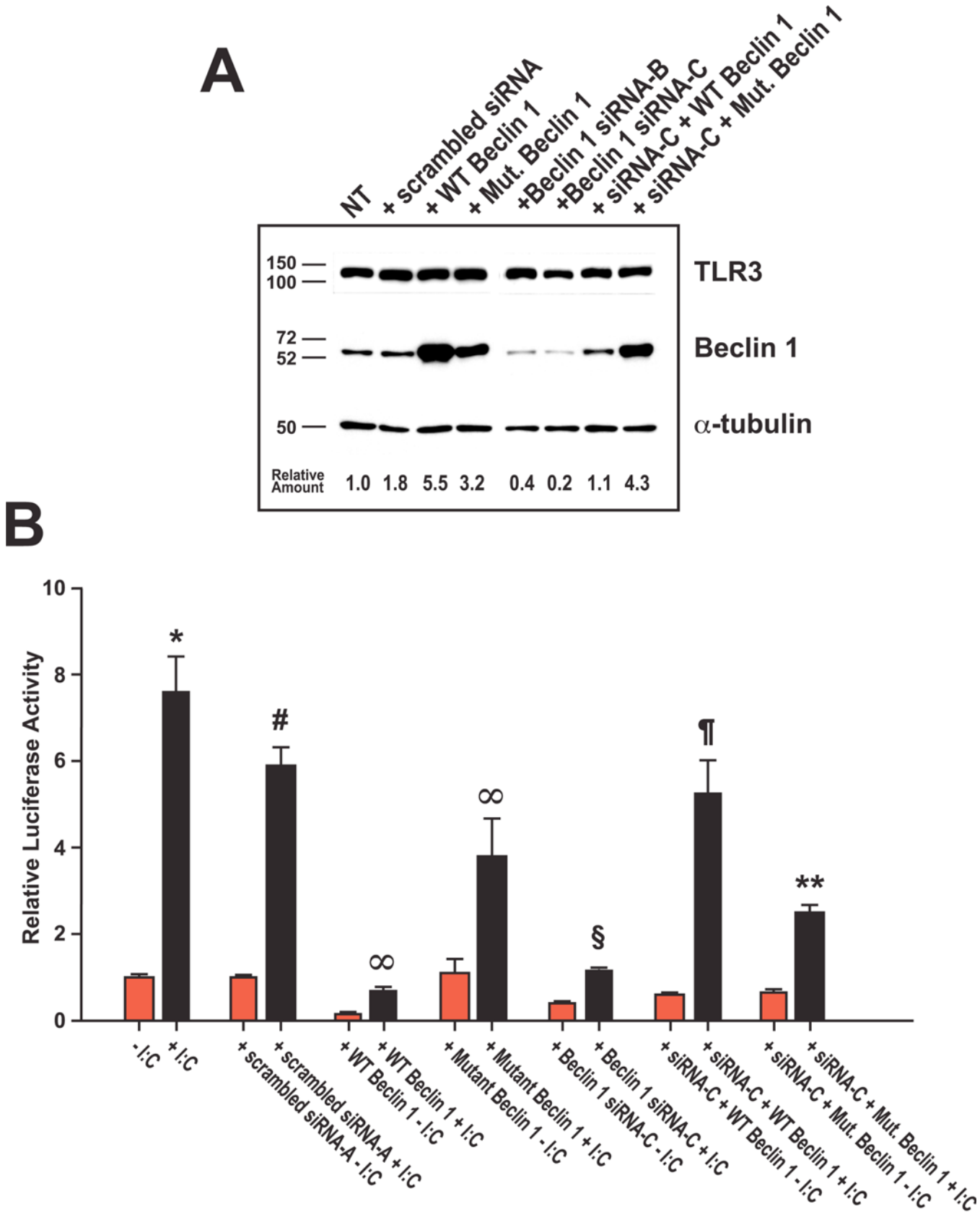
Supplementary Figure 2: TLR3 variants localize to late endosomes/multivesicular bodies (MVB). Stable COS7 cells expressing WT, Ser554 (P554S) or Phe412 (L412F) TLR3-HA were permeabilized, fixed with 3% paraformaldehyde, and stained with an antibody against HA (to detect exogenous TLR3), an affinity-purified antibody to hepatocyte growth-factor regulated tyrosine kinase substrate or Hrs (to detect MVB: a gift from Harald Stenmark), and with TO-PRO-3™ iodide (to detect nuclei). The *right panels* show the merged and magnified images of the enclosed areas directly to the left and show the partial overlap of TLR3 and Hrs staining. The *white arrows* indicate endosomes/MVB to which exogenous TLR3-HA variants and Hrs co-localize. *Bars*, full-size images: 10 μm; enlarged images: 5 μm.



Supplementary Figure 3: Gene silencing of MAP1LC3β by siRNA inhibits type I interferon signaling by TLR3 variants. *Panel A.* Levels of TLR3-HA, α-tubulin and MAP1LC3β (LC3β), with or without MAP1LC3β knockdown. Stable 293 cells expressing WT, Ser554 (P554S) or Phe412 (L412F) TLR3-HA were transfected with a scrambled siRNA control or a pool of three MAP1LC3β siRNAs, and equal amounts of protein from the whole cell extracts were then analyzed by SDS-PAGE and immunoblotting with anti-HA, anti-α-tubulin, or a polyclonal antibody to MAP1LC3β. *Panel B.* WT, Ser554 (P554S) or Phe412 (L412F) TLR3-HA expressing cells were transfected with pISRE-LUC and either a scrambled siRNA duplex, or a MAP1LC3β (siRNA-LC3β) siRNA pool, and then stimulated with poly I:C (+ I:C: *red* and *black bars*) or vehicle (*grey bars*). Relative luciferase activity in stimulated WT cells transfected with scrambled siRNA-A duplex was 9.1 ± 1.0, whereas the luciferase activity in the Ser554 and Phe412 TLR3 expressing cells was 2.3 ± 0.6 and 5.1 ± 0.6, respectively. By contrast, the luciferase activity in WT cells transfected with the MAP1LC3β siRNA pool was reduced 74% (2.4 ± 0.5), while the activity in the Ser554 and Phe412 TLR3 expressing cells decreased by 91% and 78%, respectively: 0.2 ± 0.04 in Ser554, and 1.1 ± 0.5 in Phe412. * P<0.01 *vs.* stimulated WT. ¶ P<0.03 *versus* stimulated Ser554 TLR3 cells. # P<0.01 *versus* stimulated Phe412 TLR3 expressing cells. § P<0.001 *vs*. unstimulated wild type TLR3 cells.


A
WT GFP-MAP1LC3β
Mut GFP-MAP1LC3β
WT LC3β siRNA-A
Mut LC3β siRNA-A
250
130
55
36
55
36
17
55
TLR3-HA
GFP-MAP1LC3β (anti-LC3β)
GFP-MAP1LC3β (anti-GFP)
MAP1LC3β (endogenous)
α-tubulin
Relative Amount (Total)
1.0 6.2 5.0 0.1 0.2 1.5 0.5 2.3
B
Relative Luciferase Activity
20
15
10
5
0
- I:C
+ I:C
+ WT LC3β - I:C
+ WT LC3β + I:C
+ Mut. LC3β - I:C
+ Mut. LC3β + I:C
+ siRNA-A - I:C
+ siRNA-A + I:C
+ siRNA-A + WT LC3β - I:C
+ siRNA-A + WT LC3β + I:C
+ siRNA-A + Mut. LC3β - I:C
+ siRNA-A + Mut. LC3β + I:C
+ Mut. siRNA-A - I:C
+ Mut. siRNA-A + I:C
+ Mut. siRNA-A + WT LC3β - I:C
+ Mut. siRNA-A + WT LC3β + I:C

Supplementary Figure 4: *Panel A.* Rescue of MAP1LC3β expression following transfection of plasmid encoding mutant GFP-MAP1LC3β. Stable 293 cells expressing WT TLR3-HA were subjected to RNAi using wild type LC3β siRNA-A followed by co-transfection of the siRNA duplex with a plasmid encoding either wild type or mutant GFP-MAP1LC3β fusion protein. Levels of TLR3-HA, α-tubulin and MAP1LC3β were determined by Western blotting of the whole cell extracts with anti-HA, anti-α-tubulin, and either a monoclonal antibody to GFP (to detect the GFP-MAP1LC3β fusion protein) or polyclonal anti-MAP1LC3β (to detect both endogenous and recombinant MAP1LC3β) followed by densitometry using NIH image 1.63f. The total (endogenous + recombinant) amount of MAP1LC3β in the extract is expressed relative to the level of MAP1LC3β in non-transfected cells. Transfection of either WT or mutant MAP1LC3β encoding plasmid alone increased the overall level of MAP1LC3β in the cell extracts 5- and 6-fold, respectively. Note that endogenous MAP1LC3β protein migrates on the gel near the 17-kDa marker and its level was considerably lower in cells subjected to RNAi with wild type LC3β siRNA-A, whereas the mutant GFP-MAP1LC3β fusion protein migrates between the 55- and 36-kDa markers (predicted M_r = 43 K) and restored the overall level of MAP1LC3β in LC3β siRNA-A treated cells. For comparison, we also co-transfected WT cells with a mutated (*Mut*.) version of LC3β siRNA-A and plasmid encoding wild type GFP-MAP1LC3β, in which case the overall amount of MAP1LC3β in the extract increased more than 2-fold relative to the control WT cells. The *asterisks* denote a protein that reacts non-specifically with the polyclonal antibody to MAP1LC3β. *Panel B.* Rescue of TLR3-mediated type I interferon signaling by mutant GFP-MAP1LC3β expression. WT TLR3-HA expressing cells were subjected to RNAi by transfection with wild type LC3β siRNA-A, and grown for 72 hours prior to co-transfection of the siRNA duplex with the pISRE-LUC reporter and plasmid encoding either wild type or mutant GFP-MAP1LC3β. Twenty-four hours after transfection the cells were stimulated with poly I:C (+ I:C: *black bars*) or vehicle (- I:C: *red bars*). The relative luciferase activity in WT TLR3 expressing cells transfected with reporter alone was 11.2 ± 1.7 (* $P<0.001$ *vs.* unstimulated WT), whereas transfection of either the WT or the mutant GFP-MAP1LC3β encoding plasmid alone significantly decreased interferon signaling following poly I:C stimulation to 5.5 ± 0.5 and 2.9 ± 0.3, respectively (¶ $P<0.001$ *vs*. stimulated WT; ** $P<0.01$ *vs*. stimulated WT + wild type GFP-MAP1LC3β). This indicates that high levels of exogenously expressed MAP1LC3β inhibit TLR3 signaling. The luciferase activity in WT cells transfected with reporter plus wild type LC3β siRNA-A duplex was 4.9 ± 0.5 (# $P<0.001$ *vs*. stimulated WT; $P=0.005$ *vs*. unstimulated WT), which upon expression of recombinant GFP-MAP1LC3β failed to return to the activity level present in control cells (5.4 ± 0.4: P=NS *vs*. stimulated WT + LC3β siRNA-A). In contrast, transfection of mutant GFP-MAP1LC3β encoding plasmid restored both the overall MAP1LC3β protein (*Panel A*) and signaling to the levels in WT cells transfected only with the pISRE-LUC reporter (12.8 ± 1.7: § $P<0.001$ *vs*. stimulated WT plus wild type LC3β-siRNA-A; P=NS *vs*. stimulated WT). By comparison, the luciferase activity in WT cells transfected with Mut. LC3β siRNA-A was 11.2 ± 0.2 (∞ P=NS *vs*. stimulated WT) and was reduced following transfection of both mutant duplex and wild type GFP-MAP1LC3β encoding plasmid (‡ $P<0.001$ *vs*. stimulated WT or stimulated WT cells plus mutant LC3β siRNA-A).


A
NT
+ scrambled siRNA
+ WT Beclin 1
+ Mut. Beclin 1
+Beclin 1 siRNA-B
+Beclin 1 siRNA-C
+ siRNA-C + WT Beclin 1
+ siRNA-C + Mut. Beclin 1
150
100
72
52
50
TLR3
Beclin 1
α-tubulin
Relative Amount
1.0 1.8 5.5 3.2 0.4 0.2 1.1 4.3
B
Relative Luciferase Activity
10
8
6
4
2
0
*
#
∞
∞
§
¶
**
- I:C
+ I:C
+ scrambled siRNA-A - I:C
+ scrambled siRNA-A + I:C
+ WT Beclin 1 - I:C
+ WT Beclin 1 + I:C
+ Mutant Beclin 1 - I:C
+ Mutant Beclin 1 + I:C
+ Beclin 1 siRNA-C - I:C
+ Beclin 1 siRNA-C + I:C
+ siRNA-C + WT Beclin 1 - I:C
+ siRNA-C + WT Beclin 1 + I:C
+ siRNA-C + Mut. Beclin 1 - I:C
+ siRNA-C + Mut. Beclin 1 + I:C

Supplementary Figure 5: *Panel A.* Rescue of Beclin 1 expression following transfection of plasmid encoding wild type or mutant Beclin 1. Stable 293 cells expressing WT TLR3-HA were subjected to two rounds of RNAi using Beclin 1 siRNA-C followed by co-transfection of the siRNA-C duplex with pcDNA3-Beclin 1 encoding either WT Beclin 1, or a version of the protein bearing three silent mutations within the siRNA-C sequence (*Mut. Beclin 1*). Levels of TLR3-HA, α-tubulin and Beclin 1 were determined by Western blotting of the whole cell extracts using monoclonal antibodies to the HA epitope, α-tubulin and Beclin 1. The amount of Beclin 1 in the extracts was determined by densitometry using the NIH image 1.63 software package and is expressed relative to the amount of Beclin 1 in non-transfected (*NT*) cells. For comparison, WT cells were transfected with a scrambled siRNA control, wild type or mutant Beclin 1 encoding plasmid alone (+ *WT* or + *Mut. Beclin 1*, respectively), or with Beclin 1 siRNA-B. Note that expression of mutant Beclin 1 in the presence of siRNA-C (*siRNA-C + WT Beclin 1*) resulted in a 4-fold increase in Beclin 1 expression relative to the NT control. By contrast, the level of Beclin 1 following transfection of duplex and WT plasmid (*siRNA-C + WT Beclin 1*) was unchanged relative to the NT control, and 5-fold lower than in WT TLR3 cells transfected with wild type Beclin 1 encoding plasmid. *Panel B.* Rescue of TLR3-mediated type I interferon signaling by wild type Beclin 1 expression. WT TLR3-HA expressing cells were subjected to two rounds of RNAi by transfection initially with Beclin 1 siRNA-C and then grown for 96 hours prior to co-transfection of the siRNA duplex with the pISRE-LUC reporter and either WT or mutant pcDNA3-Beclin 1. Twenty-four hours after transfection the cells were stimulated with poly I:C (+ I:C: *black bars*) or vehicle (- I:C: *red bars*). The relative luciferase activity in stimulated cells transfected with reporter alone was 7.6 ± 0.8 (* $P<0.001$ *vs.* unstimulated WT), whereas the luciferase activity in stimulated cells transfected with reporter plus scrambled siRNA control was 5.9 ± 0.4 (# P=NS *vs*. stimulated WT cells). By contrast, the luciferase activity in stimulated cells treated with siRNA-C was 1.1 ± 0.1 (~85% reduction: § $P<0.001$ *vs*. stimulated WT cells or stimulated WT plus scrambled siRNA). When WT TLR3 cells were transfected with WT or mutant Beclin 1 encoding plasmids the luciferase activity was significantly reduced upon poly I:C stimulation to 0.7 ± 0.1 and 3.8 ± 0.9, respectively (∞ $P<0.001$ *vs*. stimulated WT in both cases), indicating that high levels of exogenously expressed Beclin 1 inhibit TLR3 signaling. When the cells were transfected with both siRNA-C and the mutant Beclin 1 encoding plasmid there was no significant change in Beclin 1 expression relative to the amount of Beclin 1 detected in cells transfected with mutant plasmid alone (*Panel A*), and no significant change in luciferase activity (2.5 ± 0.2: ** P=NS *vs*. stimulated WT cells plus mutant Beclin 1). In contrast, when the cells were transfected with both siRNA-C and the wild type Beclin 1 encoding plasmid, the level of Beclin 1 expression decreased and returned to the level present in non-transfected cells (*Panel A*) and more importantly, luciferase activity was also restored and returned to the level in stimulated cells transfected with the pISRE-LUC reporter and the scrambled siRNA control (5.2 ± 0.8: ¶ P=NS *vs*. stimulated WT cells plus scrambled siRNA). Thus, the magnitude of type I interferon signaling was consistent with a similar level of Beclin 1 expression in the NT control and in the cells transfected with siRNA-C plus WT Beclin 1 encoding plasmid.