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Supplemental Material to:

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Japanese encephalitis virus replication is negatively regulated by autophagy and occurs on LC3-I- and EDEM1-containing membranes

Autophagy 2014; 10(9)
http://dx.doi.org/10.4161/auto.29455

www.landesbioscience.com/journals/autophagy/article/29455

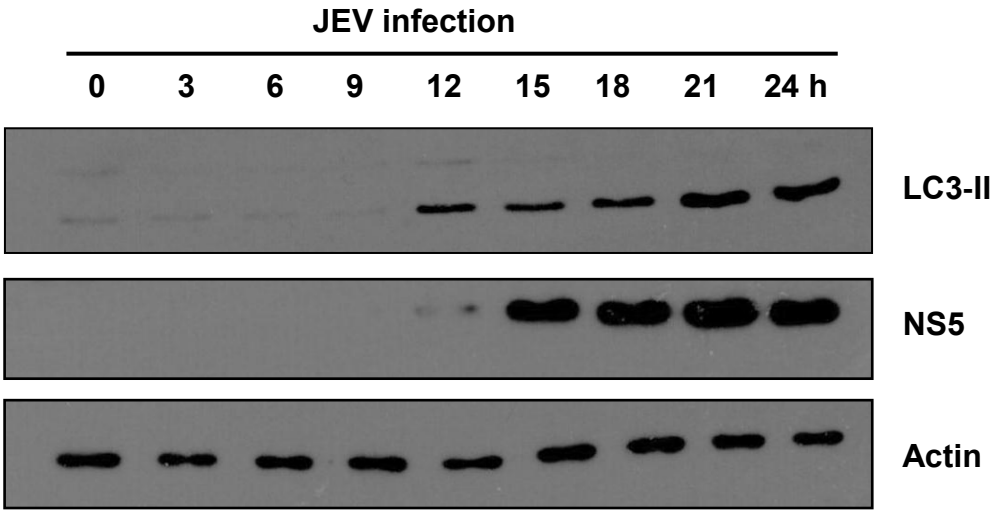

Figure S1. Time course of autophagosome accumulation in response to JEV infection. JEV-infected (MOI 5) Neuro2a cells were lysed at the indicated times pi. Protein extracts were analysed by western blotting using LC3, JEV-NS5 (infection control) and actin (loading control) antibodies.

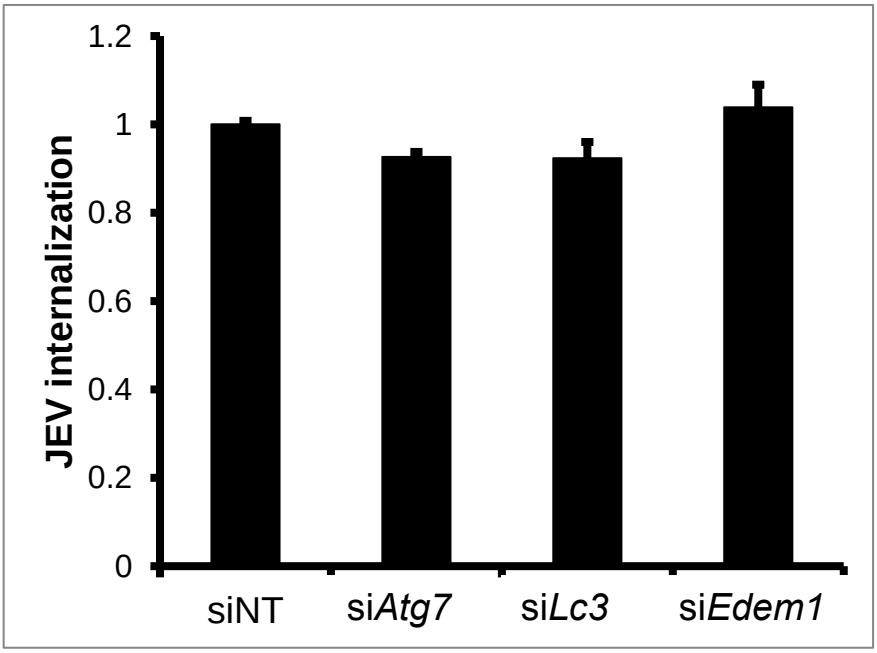

Figure S2. JEV entry is unaffected in siRNA-transfected Neuro2a cells. Neuro2a cells were transfected with control nontargeting (NT) or *Atg7*, *Lc3* (*Lc3a* and *Lc3b*), and *Edem1* siRNA for 48 h. Cells were infected with JEV (MOI 5) and the endocytosed viral load was determined 1 h pi by qRT-PCR as described in the Materials and Methods.

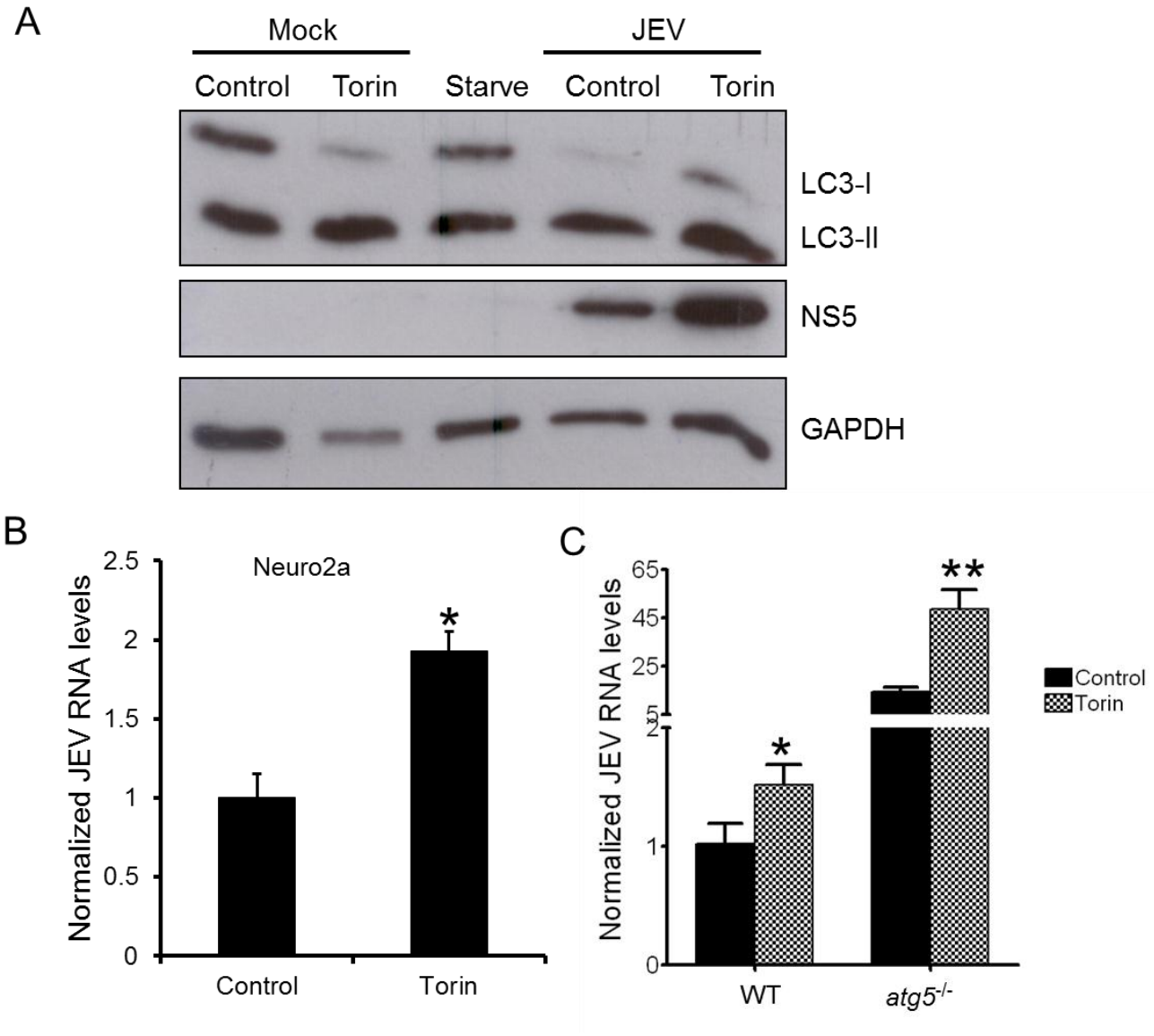

Figure S3. Torin1 enhances JEV replication independent of its effect on autophagy. (**A**) Neuro2a cells, either mock-infected or infected with JEV (MOI 5) were treated with vehicle control or Torin 1 (300 nM). As a control serum-starved cells were also taken. Cells were lysed and protein extracts were analysed by western blotting with LC3, NS5 and GAPDH (loading control) antibodies. Note that the band intensity of NS5 in JEV infected and Torin1 treated sample is much higher than in infected sample alone, indicative of increased viral protein levels. (**B and C**) Relative JEV RNA expression levels in JEV-infected, vehicle control or Torin1 treated Neuro2a cells (**B**) and WT and *atg5*$^{-/-}$ MEFs (**C**) at 24 h pi.

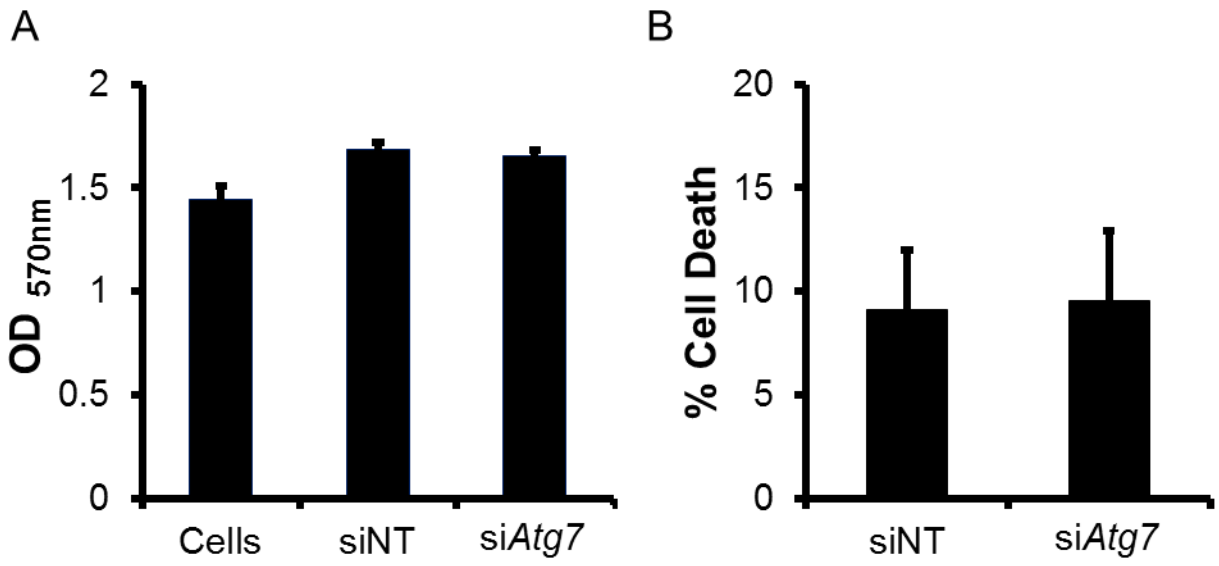

Figure S4. ATG7 depletion is not toxic and does not affect viability of Neuro2a cells. (**A**) Cell viability of Neuro2a cells, either untransfected (control) or transfected with nontargeting siRNA or *Atg7* siRNA for 48 h as determined by MTT assay. Shown is mean OD_{570nm}. (**B**) Neuro2a cells were transfected with control NT siRNA or *Atg7* siRNA for 48 h, after which they were mock-infected for 48 h. Cell death was determined by ANXA5-FITC and PI staining. Shown are mean ± standard error of values obtained from 3 independent experiments.

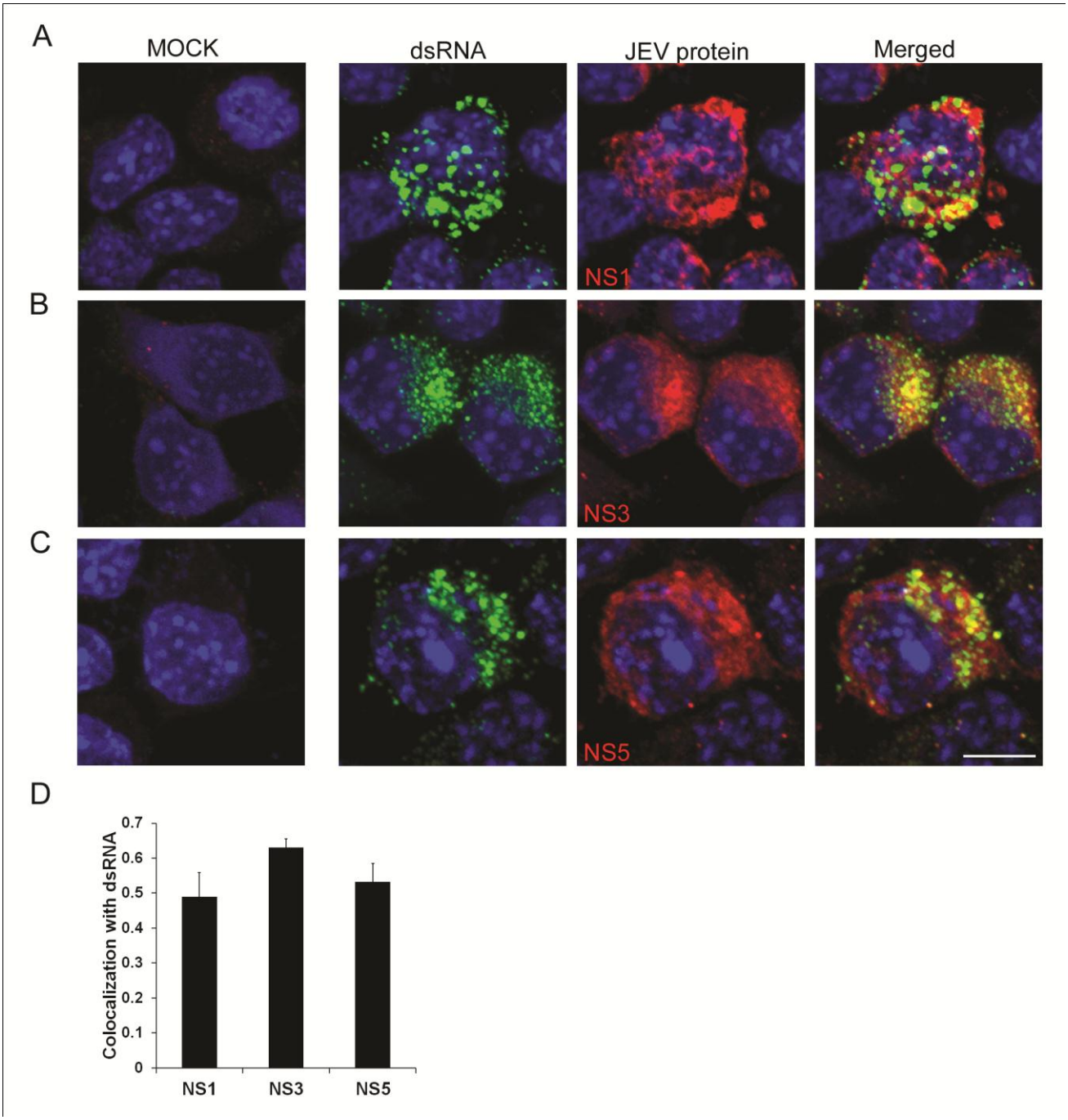

Figure S5. Viral replication complexes are marked by JEV nonstructural proteins and dsRNA. (**A-C**) Mock-or JEV-infected (MOI 5) Neuro2a cells were stained 24 h pi with mouse antibody (green) against dsRNA and rabbit antibody (red) against NS1 (**A**), NS3 (**B**), NS5 (**C**). Merging of the two signals and DAPI (blue) for nuclear staining is shown in the extreme right panels. Scale bar: 10 µm. (**D**) Bar graph showing the extent of colocalization of JEV-NS proteins with dsRNA.

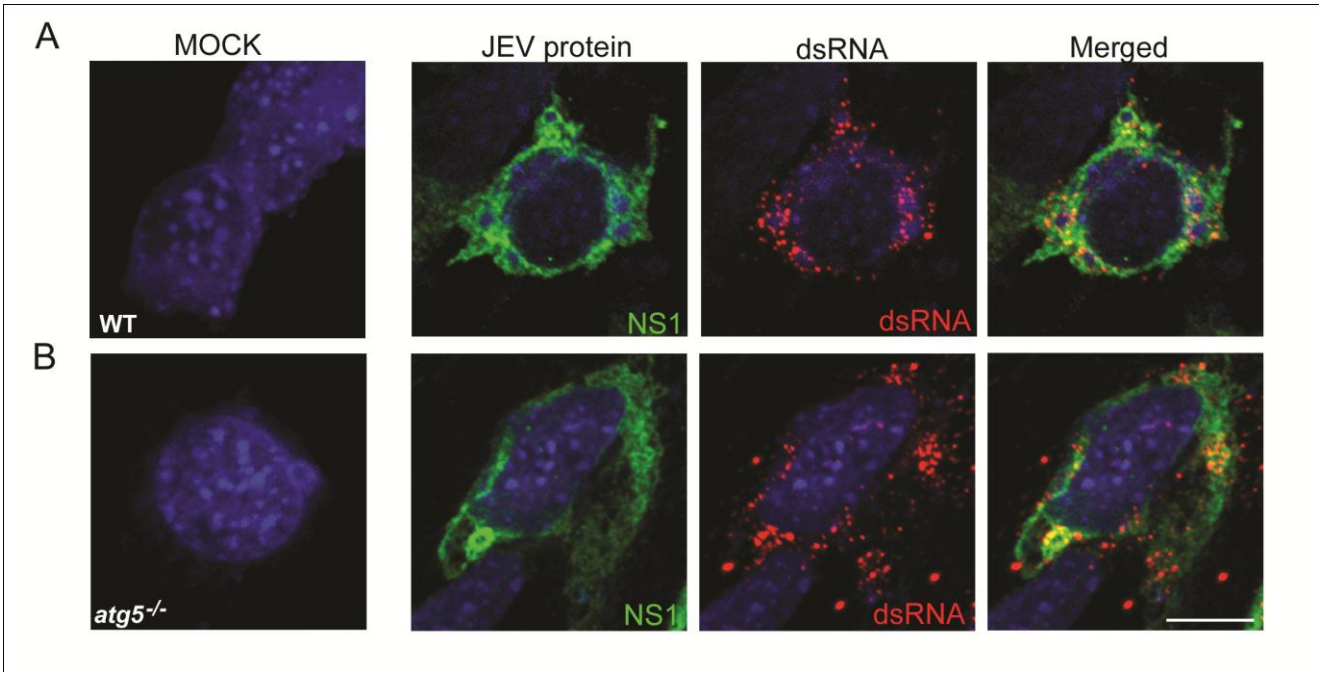

Figure S6. JEV-NS1 and dsRNA mark viral replication complexes in WT and *atg5*$^{-/-}$MEFs. WT (**A**) and *atg5*$^{-/-}$ (**B**) MEFs were mock- or JEV-infected (MOI 5) and stained 24 h pi with rabbit JEV-NS1 (green) and mouse dsRNA (red) antibodies. Merging of the two signals and DAPI (blue) for nuclear staining is shown in the extreme right panels. Scale bar: 10 µm.

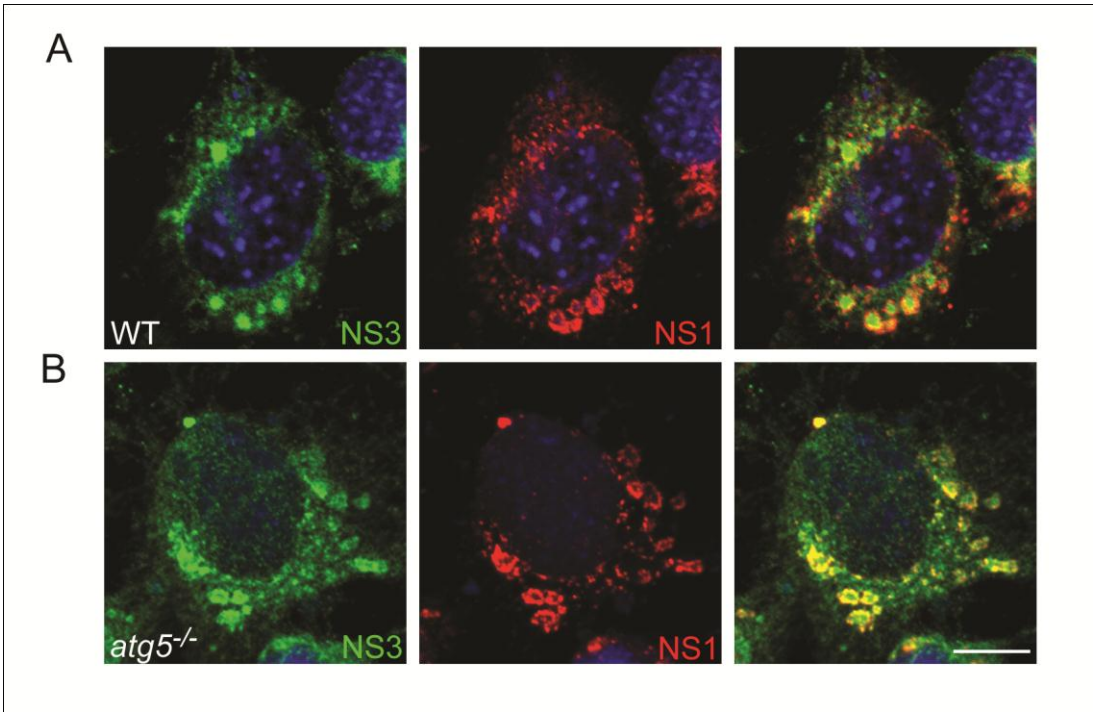

Figure S7. Intracellular distribution of JEV-NS1 and NS3 in MEFs. WT (**A**), and *atg5*$^{-/-}$ (**B**) MEF's were infected with JEV (MOI 5) and stained 24 hpi with rabbit NS3 (green) and mouse NS1 (red) antibodies. Merging of the two signals and DAPI (blue) for nuclear staining is shown in the right panels. Scale bar: 10 µm. Both antibodies show significant overlap and stain discrete puncta and vesicles that potentially represent viral replication complexes.

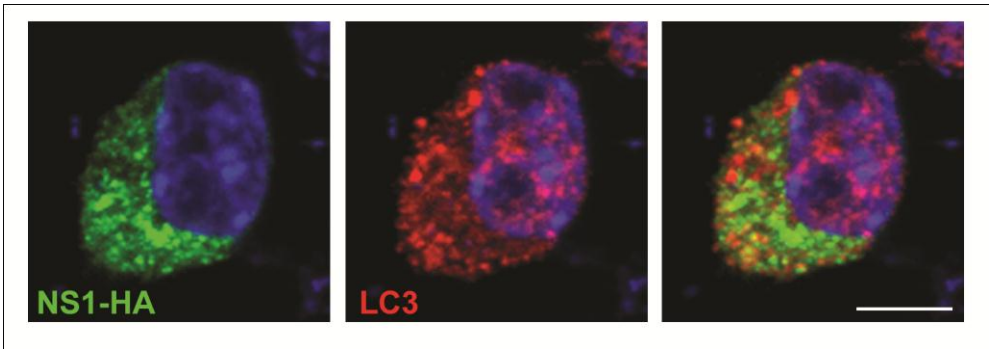

Figure S8. Overexpressed NS1 does not localize with endogenous LC3. Neuro2a cells were transfected with NS1-HA and stained with antibodies against HA-tag (green) and LC3 (red). Merging of the two signals and DAPI (blue) for nuclear staining is shown in the right panel. No significant overlap was observed between NS1-HA and LC3 (Pearson Coefficient of 0.38).

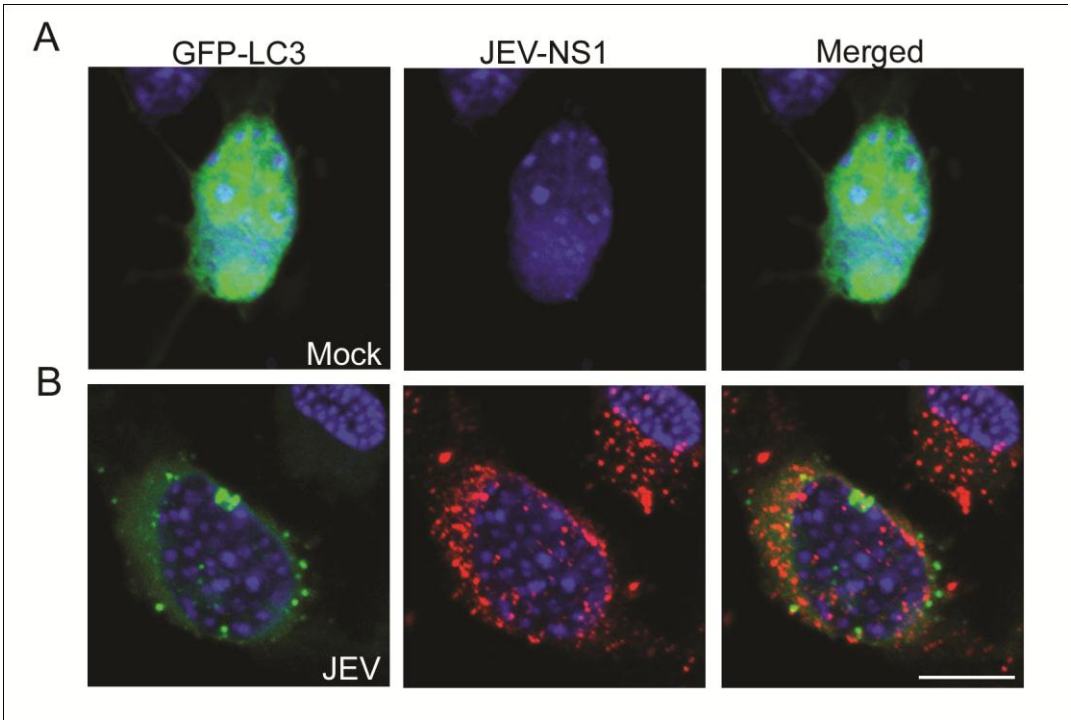

Figure S9. JEV-NS1 does not localize to GFP-LC3-positive autophagosomes in WT MEFs. GFP-LC3 (green)-transfected WT MEFs were either mock-infected (**A**), or infected with JEV (**B**). Cells were stained 24 h pi with JEV-NS1 antibody (red). Merging of the two signals and DAPI (blue) for nuclear staining is shown in the right panels. Scale bar: 10 µm. JEV-NS1 does not show any localization to GFP-LC3-positive structures.

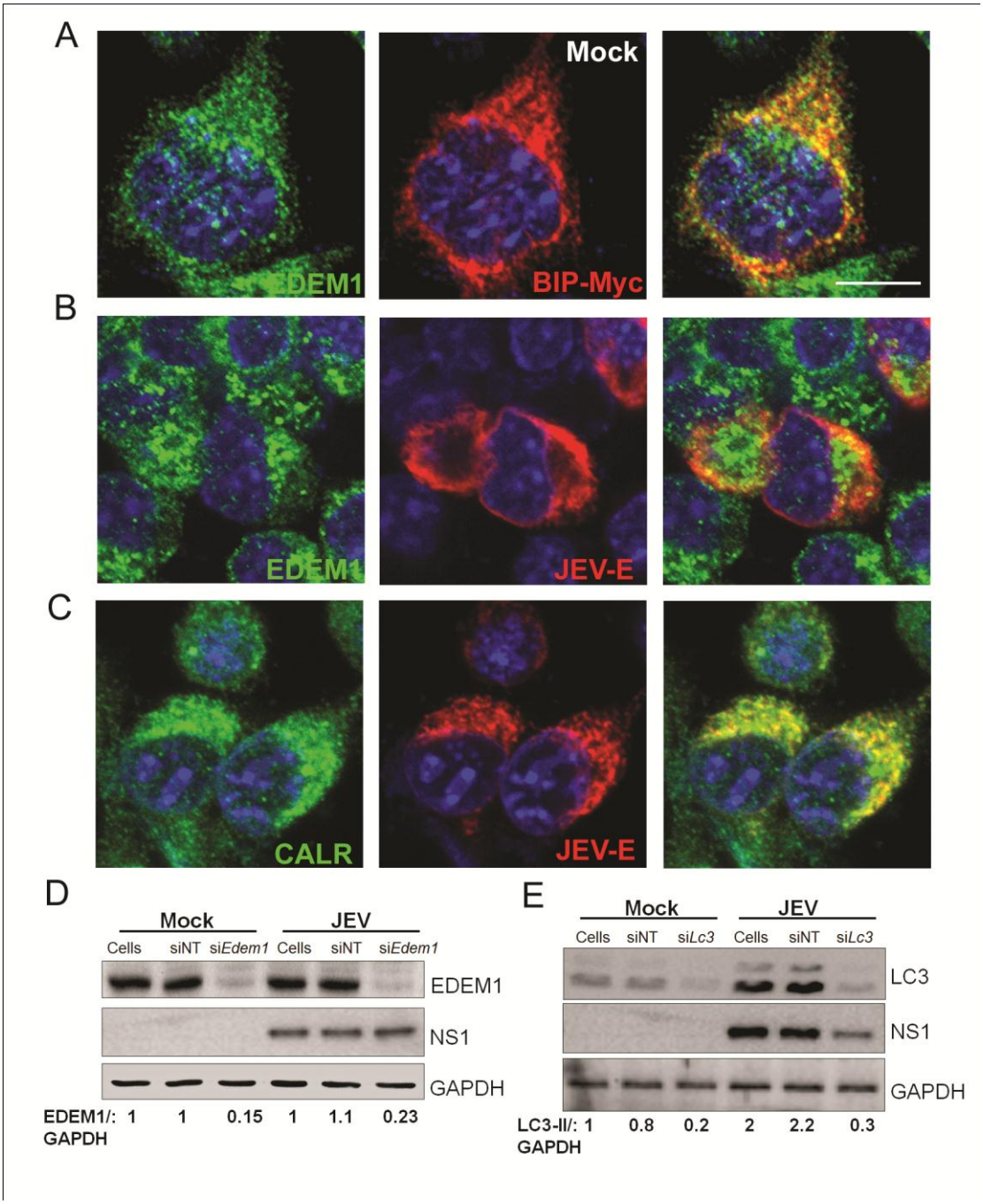

Figure S10: EDEM1 localization in Mock- and JEV-infected cells. (**A**) Neuro2a cells transfected with BIP-Myc were mock-infected and stained with EDEM1 (green) and Myc (red) antibodies. (**B and C**) JEV (MOI 5) infected Neuro2a cells were stained with EDEM1 and JEV-E (**B**), or CALR and JEV-E (**C**) antibodies. Merging of the two signals is shown in the right panels. Scale bar: 10µm. (**D and E**) Neuro2a cells were either untransfected or transfected with NT siRNA or *Edem1* siRNA (**D**) or *Lc3* (*Lc3a* and *Lc3b*) siRNA (**E**), and were either mock- or JEV-infected (MOI 5) for 24 h. Cell lysates were analysed by western blotting with EDEM1, LC3, NS1 and GAPDH (loading control) antibodies.

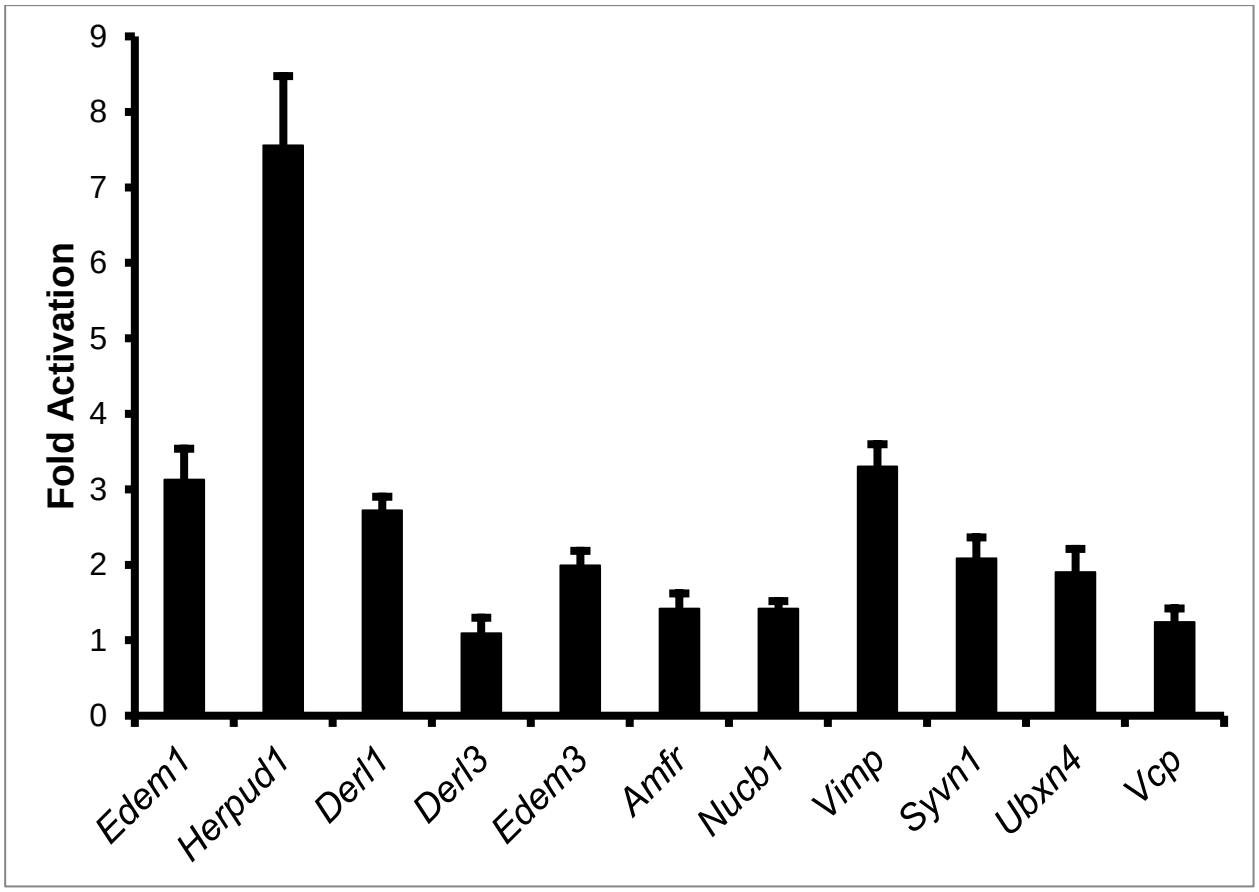

Figure S11: JEV infection leads to transcriptional upregulation of genes involved in the ERAD pathway. Quantitiative PCR (qPCR) of ERAD pathway genes in JEV infected Neuro2a cells. Graph shows the relative increased expression of gene transcription normalized to mock-infected samples. Values represent mean ± SD of 3 independent experiments.