

Expanded View Figures

Figure EV1. Phosphorylation of STING and STING signalling molecules.

- A Schematic of the C-terminal tail (CTT) of STING where multiple phosphorylation sites were identified.
- B–L The measured intensity values from the phosphoproteomics screen for phosphorylated peptides corresponding to STING (B–E), IKK β (F), CYLD (G–H), OPTN (I), iRHOM2 (J–L), were plotted for UT, DMSO and DMXAA-treated conditions. The phosphorylated residue is indicated within the graph title. Data shown as mean $\pm$ SEM for four replicates. Statistical analysis was performed using unpaired Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

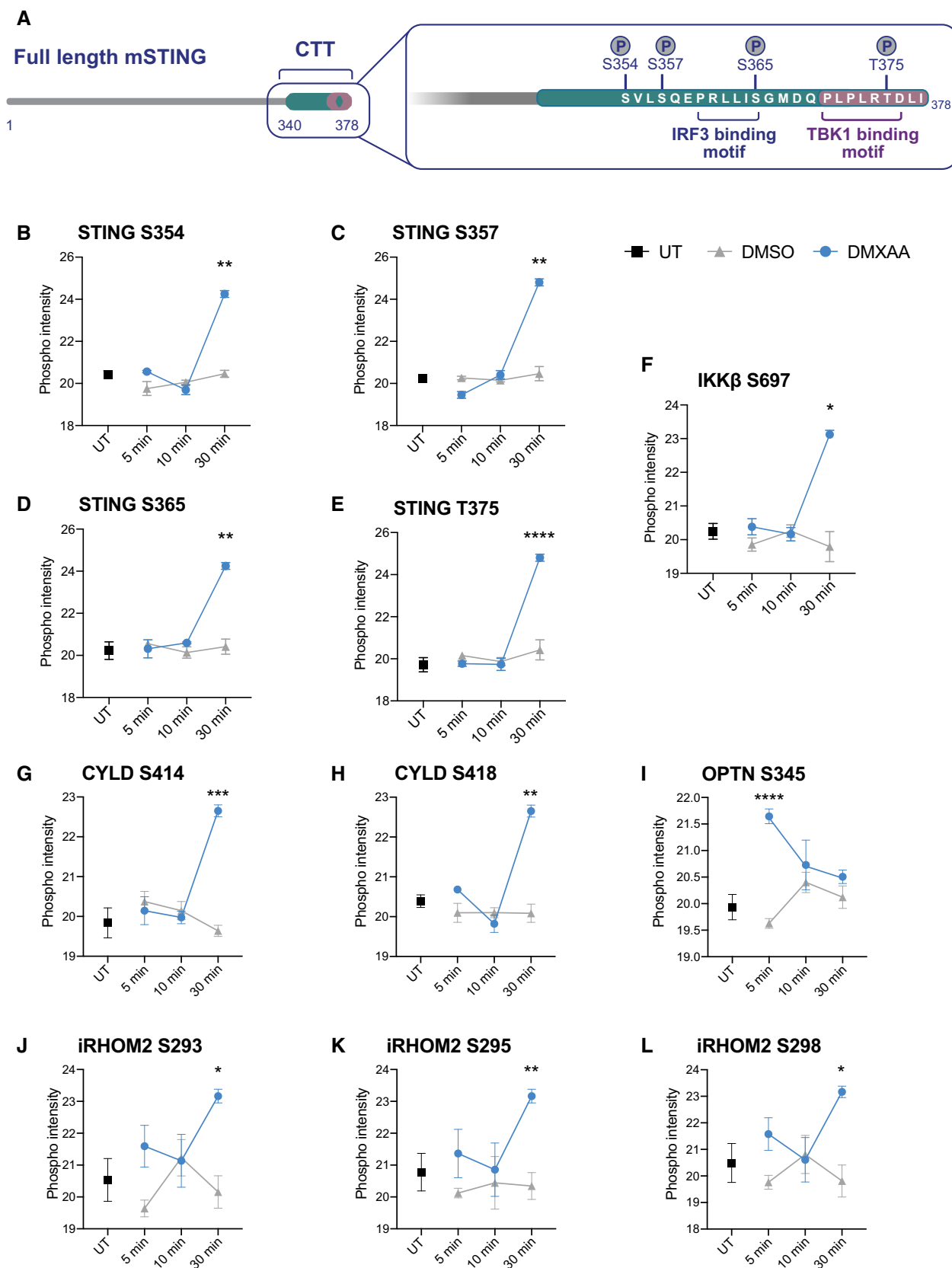


Figure EV1.

Figure EV2. Phosphorylation of trafficking proteins in response to STING activation.

- A–F The measured intensity values from the phosphoproteomics screen for phosphorylated peptides corresponding to Rab7a (A), CHMP4B (B), LYST (C), TOM1 (D), ATP6V1G3 (E) and CD-MPR (F) were plotted for UT, DMSO and DMXAA-treated conditions. The phosphorylated residue is indicated within the graph title. Data shown as mean $\pm$ SEM for four replicates. Statistical analysis was performed using unpaired Student's *t*-test. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.
- G Gene ontology (GO) analysis was performed for all proteins found with phosphorylation changes across all three timepoints. Proteins characterised within the organelles STING traffics through were identified using "Cellular components". Proteins localised to the Golgi, endosomes, lysosomes and clathrin-coated vesicles are depicted on a schematic of STING trafficking.

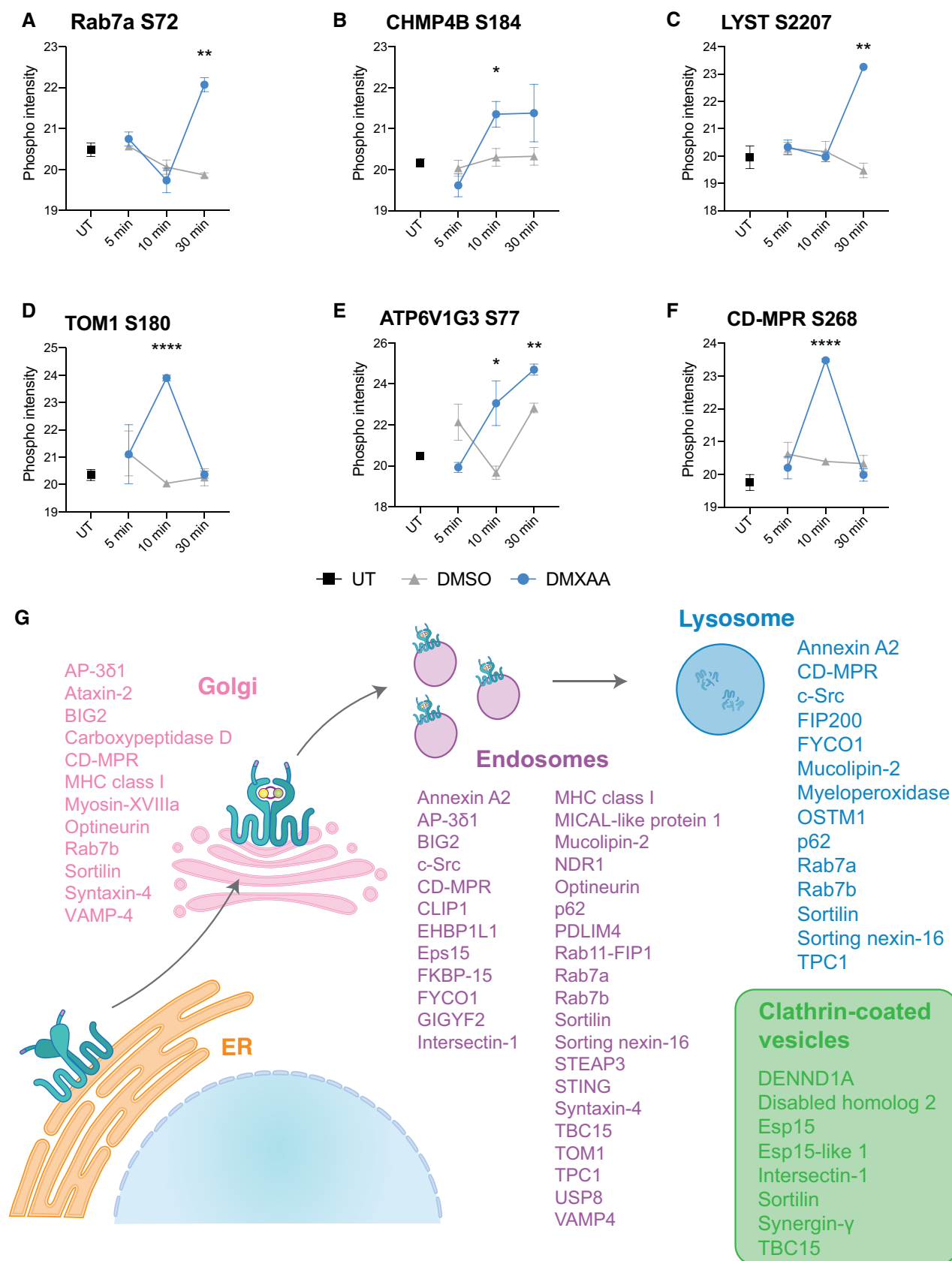


Figure EV2.

Figure EV3. STING rapidly traffics from the ER to the Golgi.

- A *Sting*^{-/-} iBMDMs alone or *Sting*^{-/-} iBMDMs reconstituted with either eGFP- or mRuby3-tagged STING were treated with 50 µg/ml BMDMs for 2 h. Cells were lysed for immunoblot with the indicated antibodies. Data shown are representative of three independent experiments.
- B *Sting*^{-/-} iBMDMs expressing eGFP-STING were imaged using spinning disk microscopy in resting conditions. Data are shown as both a single Z slice and maximum intensity projection (MIP) of Z stack images. Data shown are representative of three independent experiments. Scale bar = 5 µm.
- C *Sting*^{-/-} iBMDMs expressing eGFP-STING were imaged live on the spinning disk microscope. Images display a time series as indicated, showing STING translocation from ER to Golgi after 50 µg/ml DMXAA treatment. Data are shown as both a single Z slice and MIP of Z stack images. Data shown are representative of three independent experiments. Scale bar = 5 µm. Corresponds to Movie EV2.
- D *Sting*^{-/-} iBMDMs expressing eGFP-STING were imaged live on the spinning disk microscope. Images display a time series as indicated, showing STING vesicles exiting the Golgi for a recording starting 30 min after 50 µg/ml DMXAA treatment. Data are shown as a MIP of Z stack images. Data shown are representative of three independent experiments. Scale bar = 5 µm. Corresponds to Movie EV5.

Source data are available online for this figure.

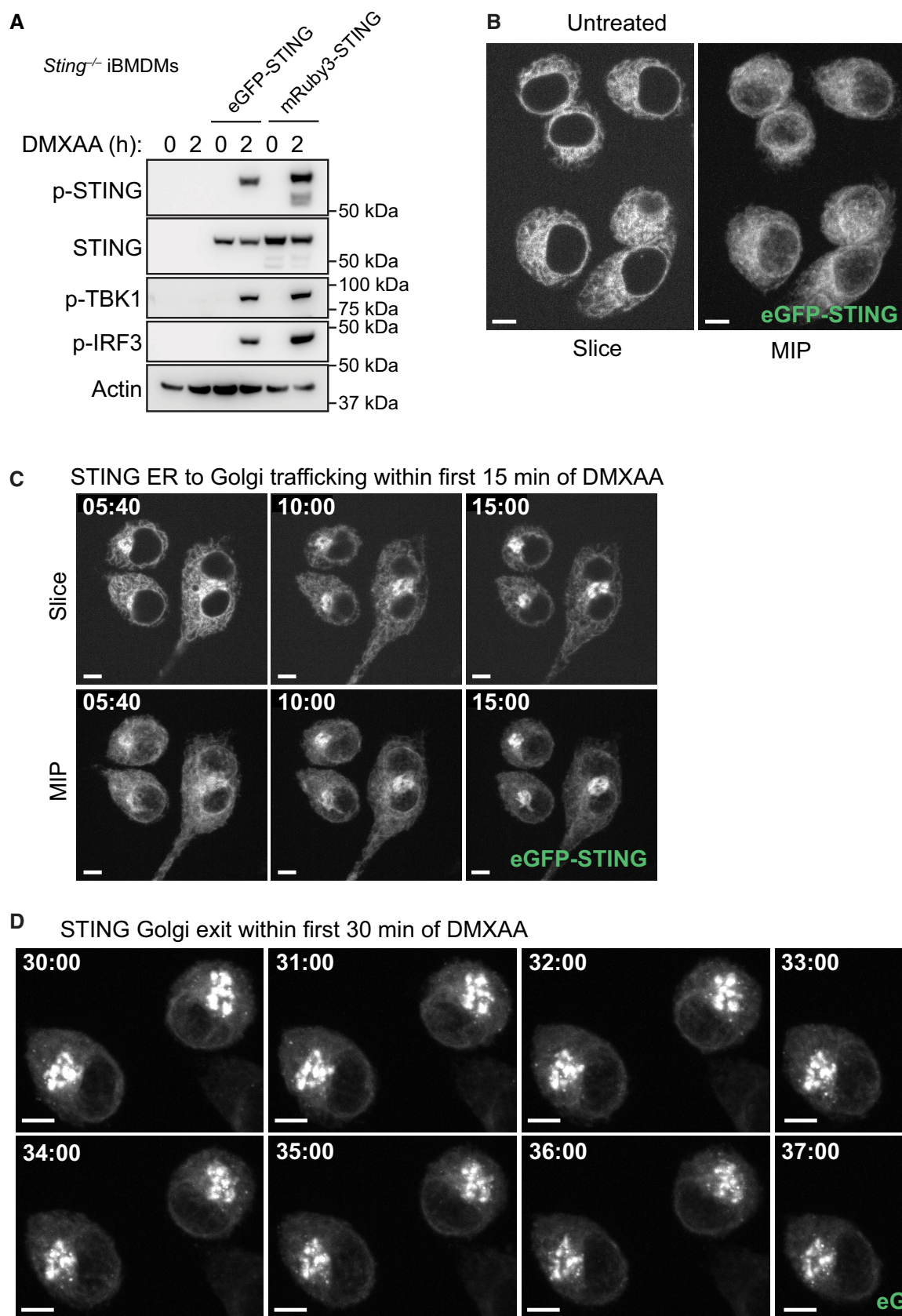


Figure EV3.

Figure EV4. STING interacts with HRS.

- A WT or TBK1/IKK ϵ^{dKO} iBMDMs were left untreated (i.e. 0 h) or treated with 50 μ g/ml DMXAA for 1 or 3 h. Cells were lysed and a portion of lysate underwent immunoblot with the indicated antibodies (i.e. lysate). The remaining lysate was incubated with TUBE beads to isolate ubiquitinated proteins (i.e. TUBE). TUBE samples underwent immunoblot with the indicated antibodies. Data representative of three independent experiments.
- B *Sting*^{-/-} iBMDMs or *Sting*^{-/-} iBMDMs expressing HA-STING were left untreated or treated with 50 μ g/ml DMXAA for 3 h. Cells were lysed and a portion of lysate underwent immunoblot with the indicated antibodies (i.e. lysate). The remaining lysate underwent immunoprecipitation with an anti-HA antibody (i.e. HA pull down). Samples then underwent immunoblot with the indicated antibodies. Data representative of three independent experiments.
- C Primary BMDMs were left untreated or treated with 50 μ g/ml DMXAA for 3 h. Cells were lysed and a portion of lysate underwent immunoblot with the indicated antibodies (i.e. lysate). The remaining lysate underwent immunoprecipitation with an anti-STING antibody (i.e. STING pull down). Samples then underwent immunoblot with the indicated antibodies. Data representative of three independent experiments.
- D *Sting*^{-/-} iBMDMs expressing eGFP-STING (yellow) were treated with 50 μ g/ml DMXAA for 3 h. Cells were fixed and underwent immunofluorescence staining for conjugated ubiquitin (Ub; magenta) and HRS (cyan). Data show two channel merged images relating to those found in Fig 5D. Scale bar = 5 μ m.

Source data are available online for this figure.

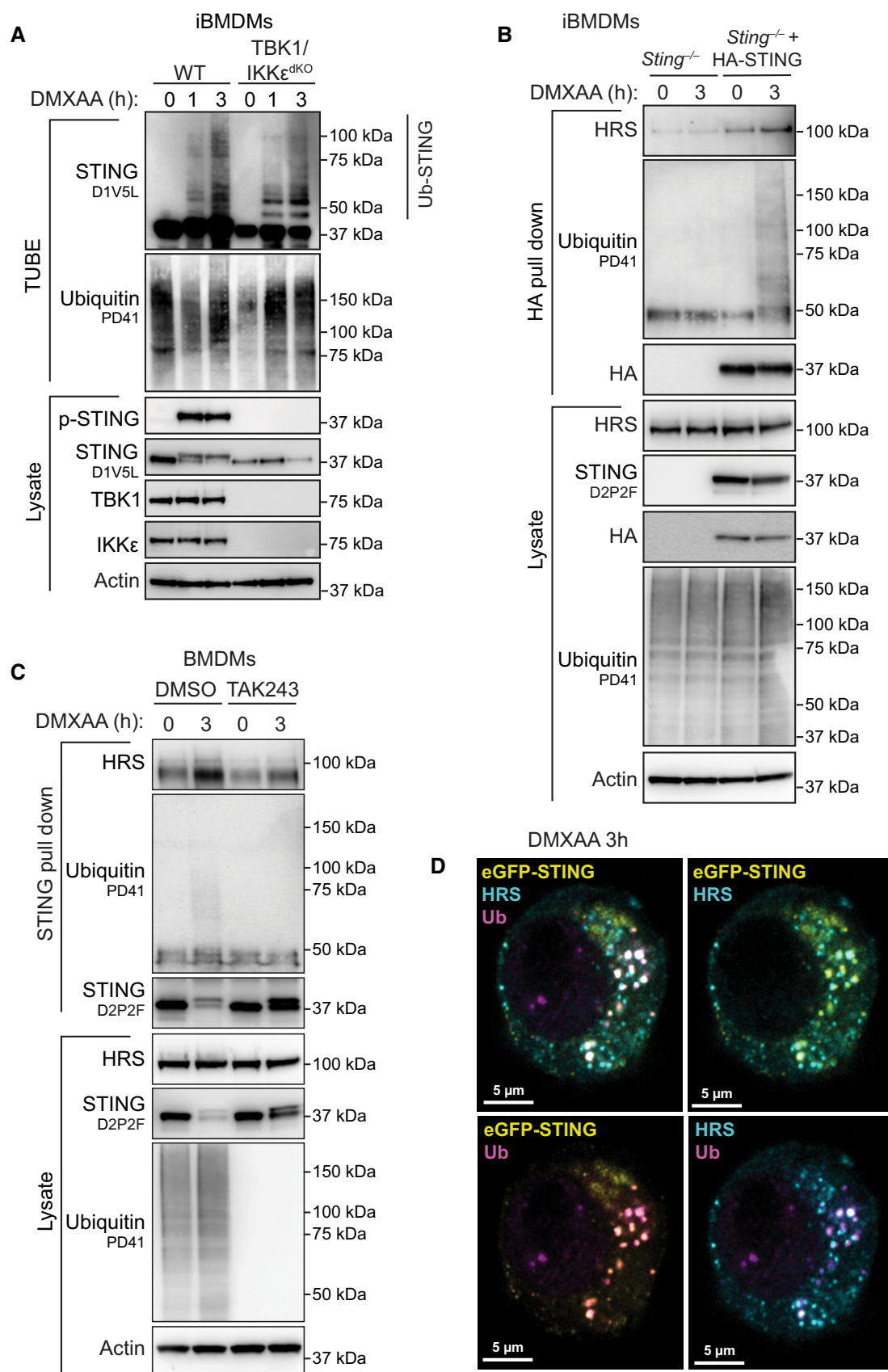


Figure EV4.

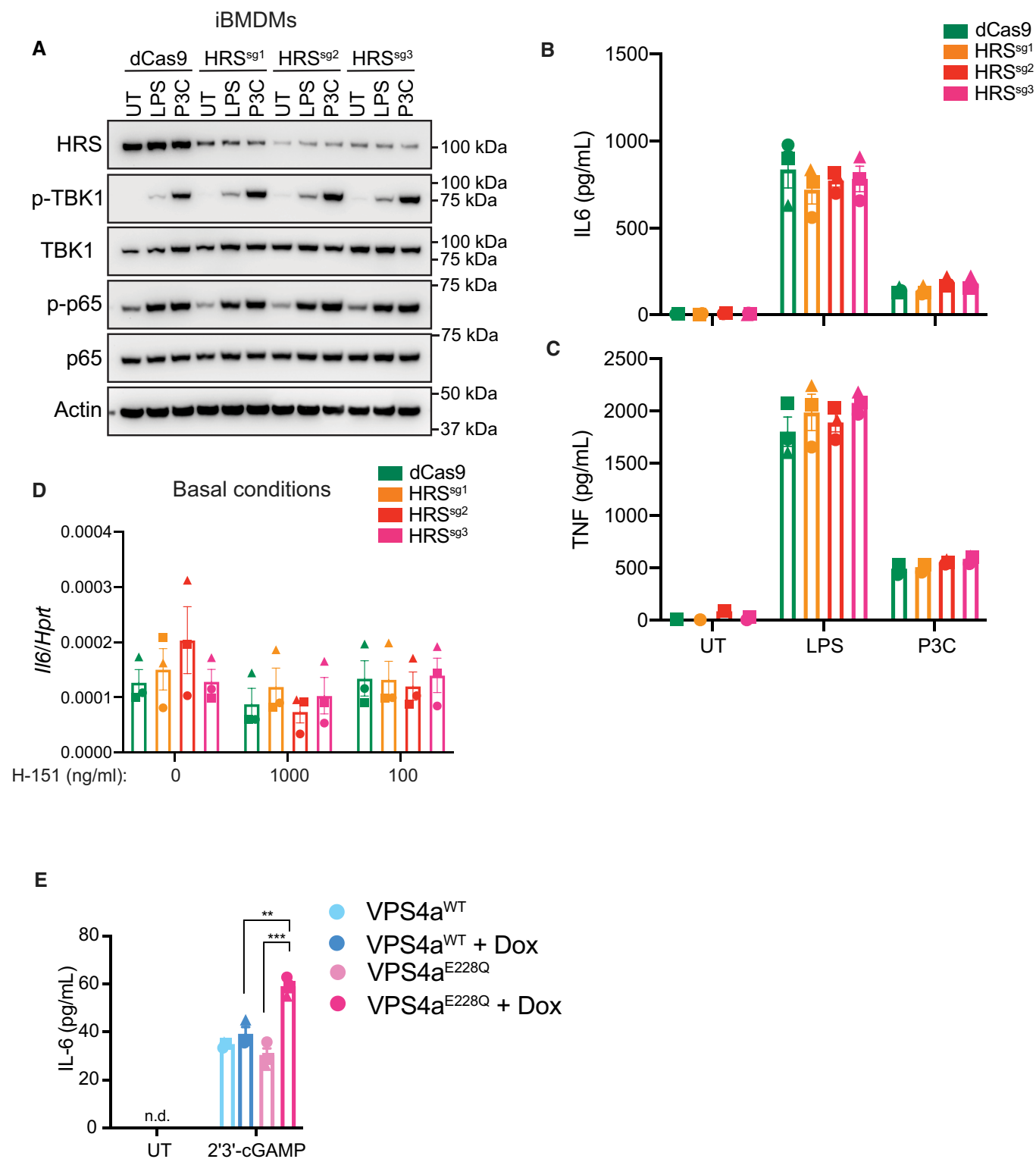


Figure EV5.

Figure EV5. HRS-depleted iBMDMs maintain normal TLR responses.

- A–C dCas9-KRAB expressing iBMDMs, without (i.e. dCas9 alone) or with HRS-targeting sgRNAs (i.e. sg1, sg2 or sg3) were treated for 48 h with 1 µg/ml doxycycline (Dox) to induce sgRNA expression. Cells were then left untreated (UT), or treated with 500 ng/ml LPS or 200 ng/ml Pam3CSK4 (P3C) for 6 h. A Cells were lysed for immunoblot with the indicated antibodies. Data shown are representative of three independent experiments. (B, C) Cell supernatant was collected and assayed for secreted IL-6 (B) or TNF (C) by ELISA. Data are shown as mean ± SEM combined from $N = 3$ independent experiments. Statistical analysis was performed using two-way ANOVA using Bonferroni's multiple comparisons test, where no statically significant differences were observed across cell lines for the same treatment.
- D dCas9-KRAB expressing iBMDMs, without (i.e. dCas9 alone) or with HRS-targeting sgRNAs (i.e. sg1, sg2 or sg3) were treated for 24 h with 1 µg/ml Dox to induce sgRNA expression. iBMDMs were further treated with 1 µg/ml (i.e., 1,000 ng/ml) or 100 ng/ml H-151 as indicated and incubated for a further 24 h. Cells were lysed for RNA purification and the expression of *Il6* was analysed by qPCR. Data are shown as mean ± SEM combined from $N = 3$ independent experiments. Statistical analysis was performed using two-way ANOVA using Bonferroni's multiple comparisons test, where no statistically significant changes were observed.
- E VPS4a^{WT} or VPS4a^{E228Q} dominant negative BMDMs were left UT or treated with 1 µg/ml Dox for 4 h. iBMDMs were then further left UT or treated with 10 µg/ml 2'3'-cGAM(PS)2 for 4 h. Cell supernatant was collected and secreted IL-6 was measured by ELISA. Data are shown as mean ± SEM combined from $N = 3$ independent experiments. Statistical analysis was performed using one-way ANOVA using Bonferroni's multiple comparisons test, where $**P < 0.01$, $***P < 0.001$. n.d., not detected.

Source data are available online for this figure.