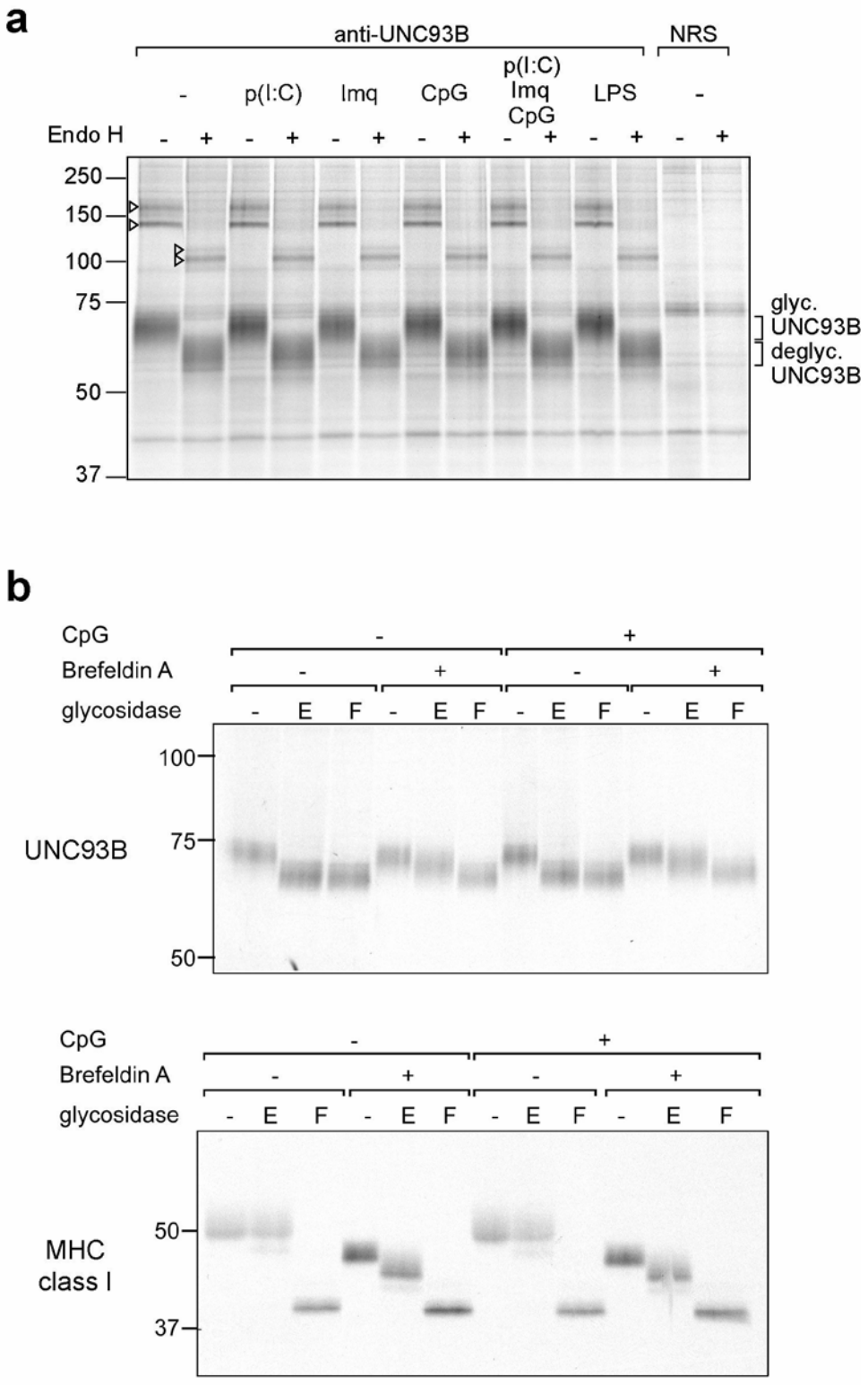
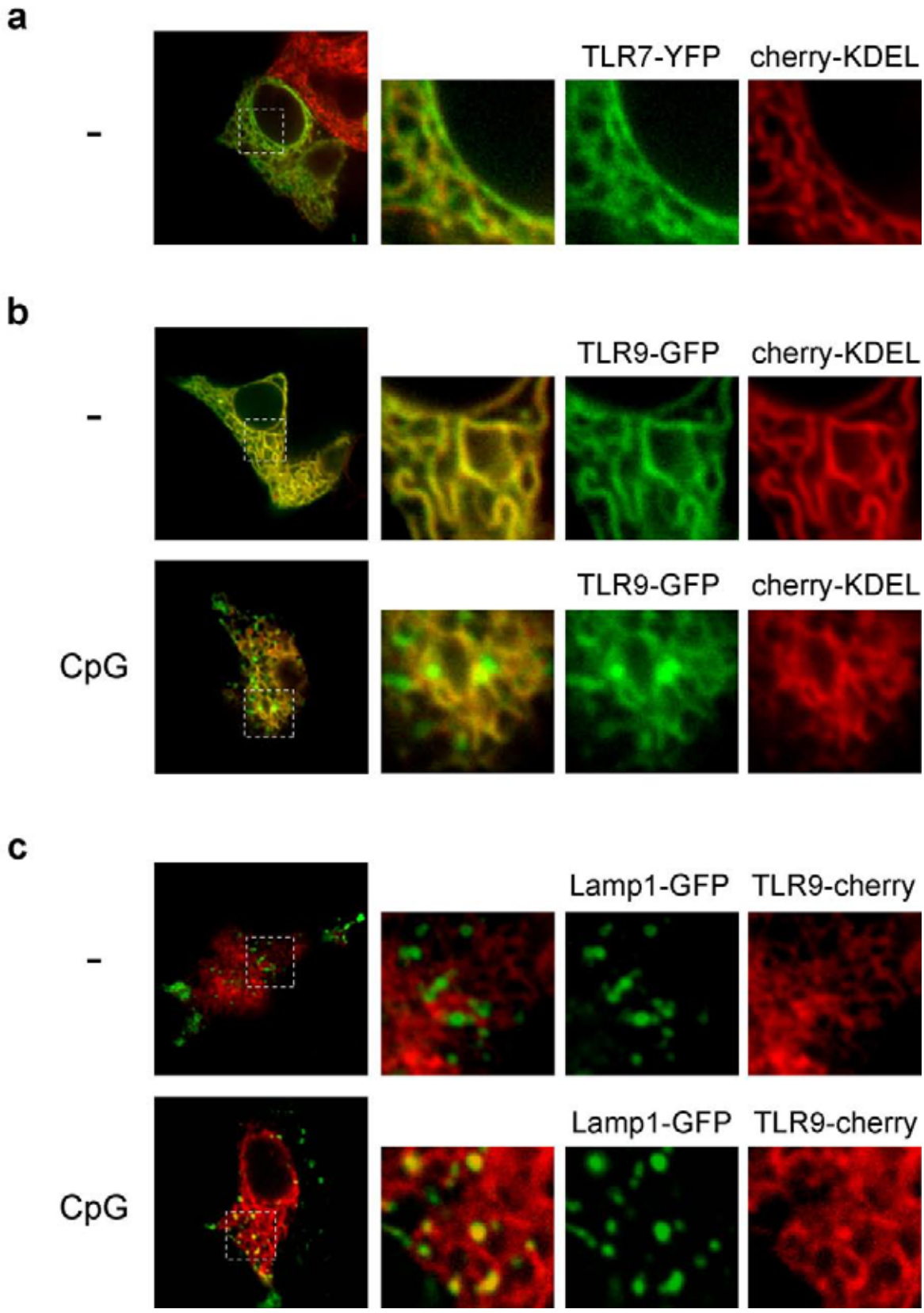
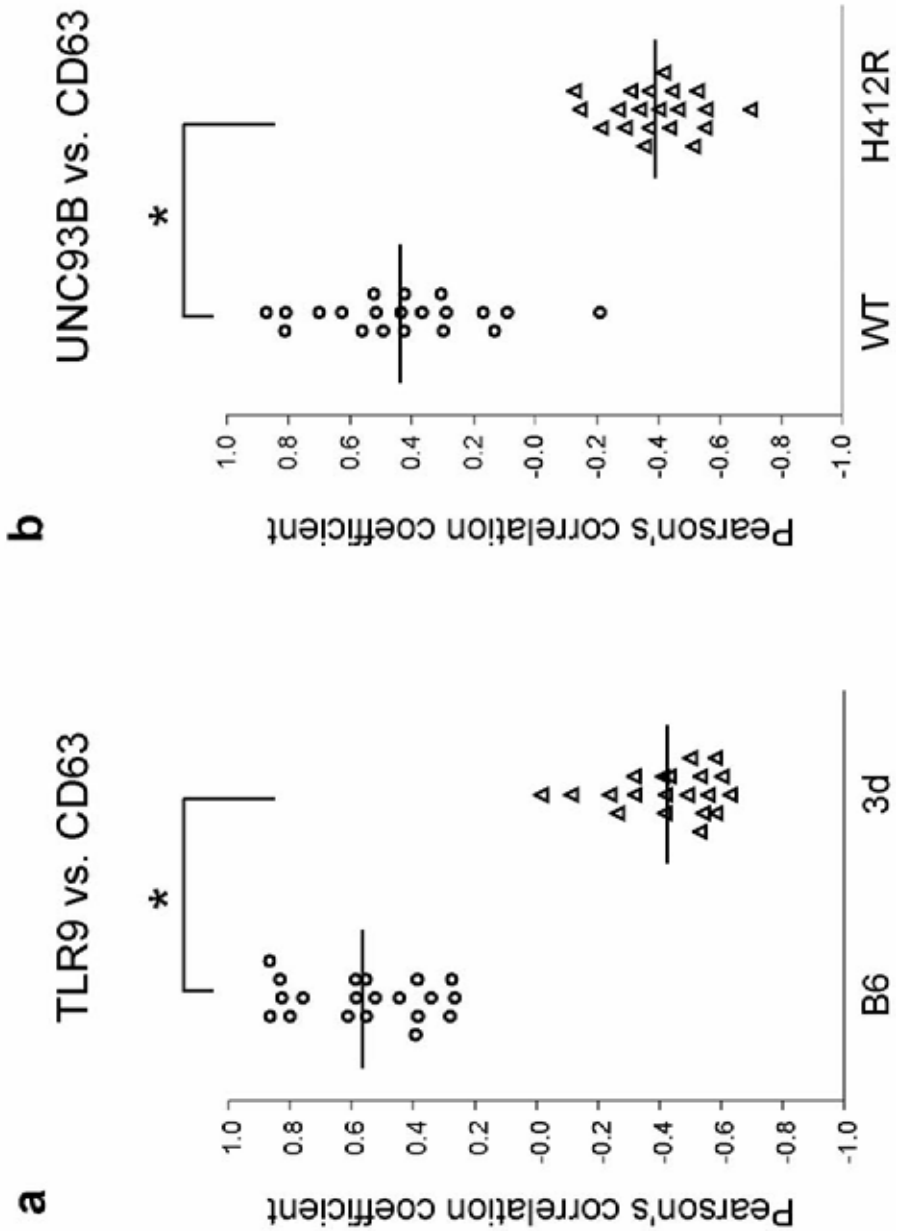
Supplementary Fig. 1



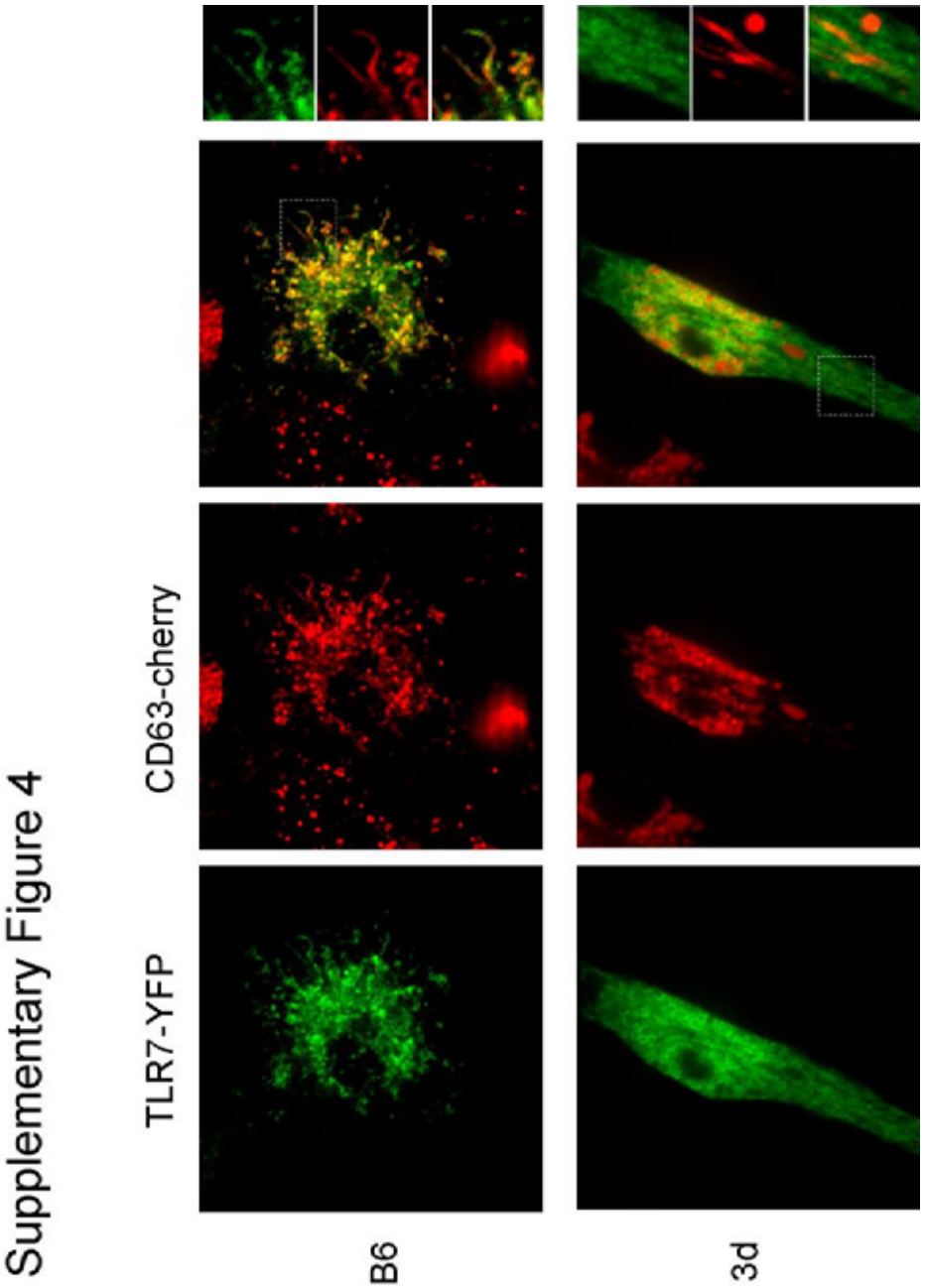
Supplementary Figure 2



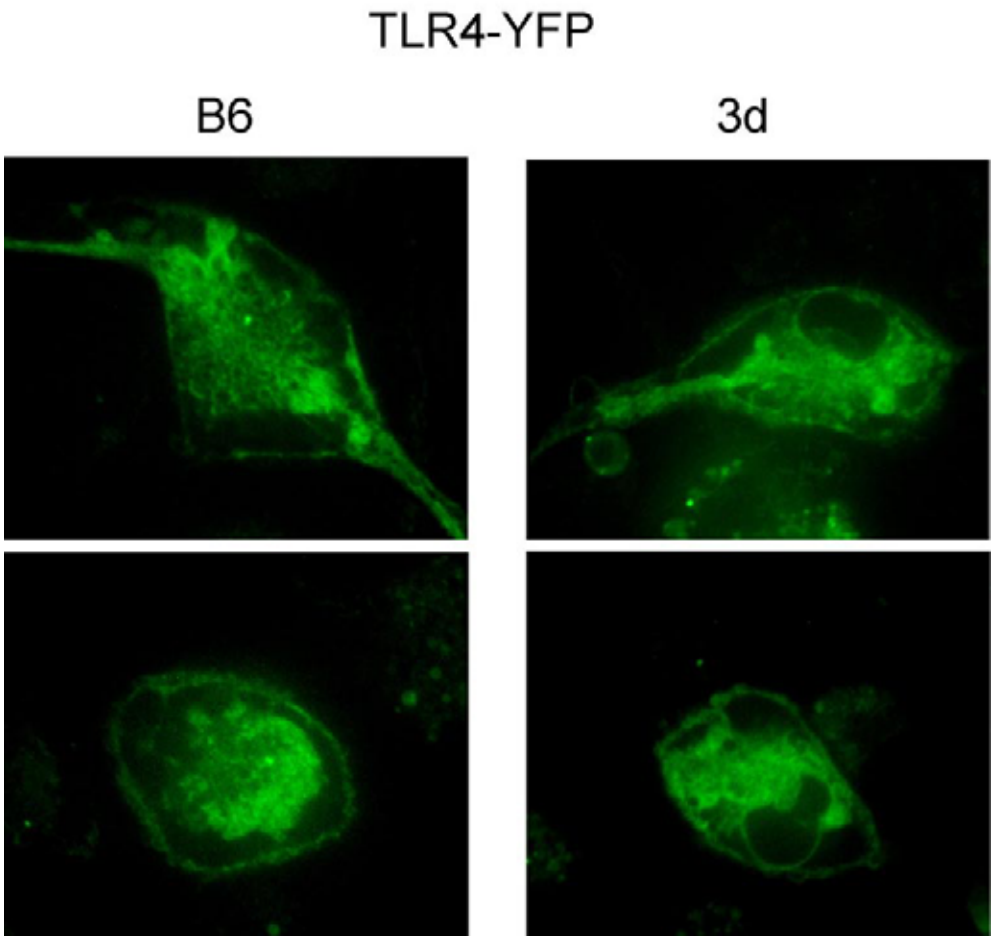
Supplementary Fig. 3



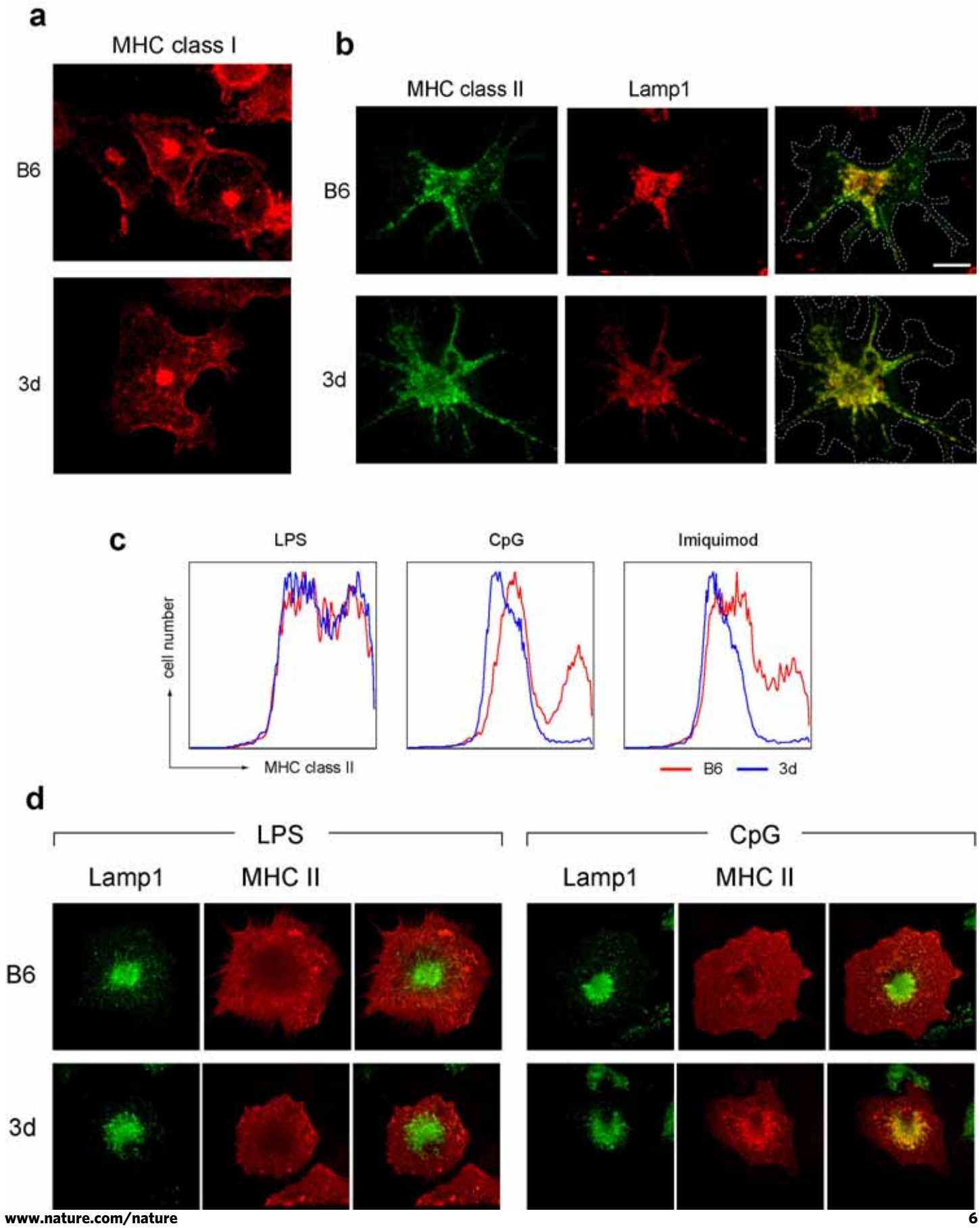
Supplementary Figure 4



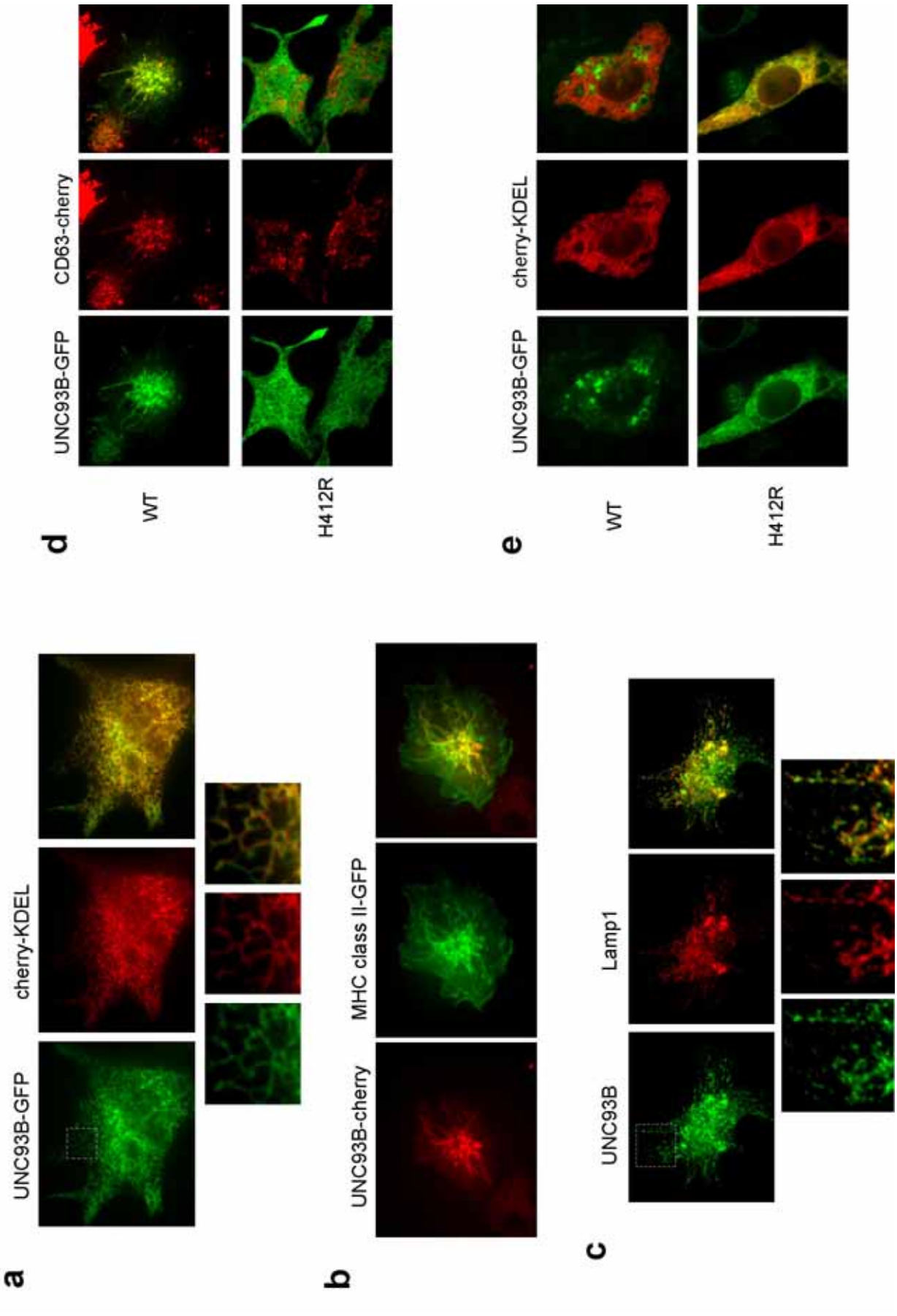
Supplementary Figure 5



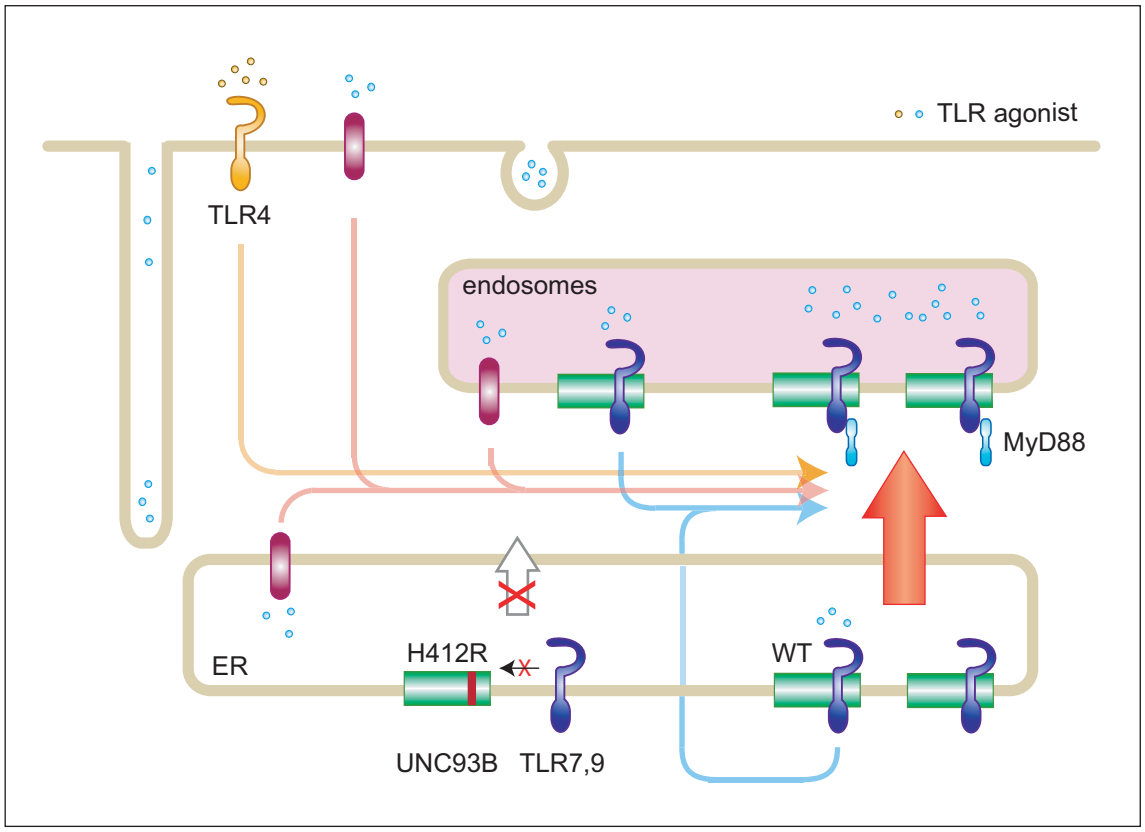
Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure Legends

Supplementary Figure 1. The interaction between UNC93B and TLRs maintains in dendritic cells stimulated with TLR agonists.

(**a**) BM-DCs were metabolically labeled with ^{35}S- methionine/cysteine and stimulated with TLR ligands for 1 h (LPS: 1 μg/ml, poly (I:C): 50 μg/ml, Imiquimod: 2.5 μM, CpG DNA: 2.5 μM). Endogenous UNC93B and TLRs were immunoprecipitated with an anti-UNC93B antibody, digested with endoglycosidase H (Endo H), subjected to 10% SDS–PAGE and visualized by fluorography. TLRs coimmunoprecipitated with UNC93B are denoted by open triangles. Interaction between TLRs and UNC93B was maintained even after TLR stimulation with various agonists. Neither UNC93B nor TLRs aquire resistance to Endo H digestion, which indicates that they are not exposed to carbohydrate modifying enzymes in the Golgi apparatus. NRS; normal rabbit serum (**b**) RAW macrophages stably expressing wild type UNC93B-HA were exposed to Brefeldin A (10 μg/ml) for 2 h, metabolically labeled with ^{35}S-methionine/cysteine for 30 min (pulse), incubated in normal medium for 2 h (chase) followed by 2 h stimulation with CpG (+CpG) or were left unstimulated (-CpG). All incubations (pulse, chase, CpG stimulation) were done in the continuous presence of Brefeldin A. As a control, cells were not treated with Brefeldin A (-BA). Cells were lysed in RIPA buffer and UNC93B-HA and Class I MHC products were recovered from cell lysates by immunoprecipitation with an anti-HA (upper panel) or Class I antibody (lower panel), respectively. Re-immunoprecipitation was carried out with an anti-UNC93B antibody (upper panel) or Class I antibody (lower panel) after denaturation of the immunoprecipitates. Recovered UNC93B-HA and Class I MHC were subjected to digestion with glycosidases Endo H (E) or PNGase F (F) or not digested (-). Normally, UNC93B retains full sensitivity to Endo H digestion, irrespective of stimulation with TLR agonists, whereas surface disposed Class I MHC products acquire EndoH resistance. In the presence of Brefeldin A, which disrupts the Golgi apparatus and relocalizes Golgi-resident proteins to the ER, both Class I MHC and UNC93B acquire partially EndoH resistant hybrid type oligosaccharides.

Supplementary Figure 2. TLR7 and TLR9 are localized in the ER of unstimulated cells and translocate to endosomes upon stimulation of cells.

TLR7-YFP (**a**), TLR9-GFP (**b**) or TLR9-cherry (**c**) were coexpressed with either cherry-KDEL (**a**,**b**) or Lamp1-GFP (**c**) in HEK-293T cells by transient transfection. After 48 h, live cells were imaged either

unstimulated (-) or stimulated with 1 μM CpG for 2 h (CpG). Both TLR7-YFP and TLR9-GFP colocalize with the ER marker cherry-KDEL in unstimulated cells. In CpG-stimulated cells, a fraction of TLR9-GFP does not colocalize with cherry-KDEL, suggesting that they are exported from the ER. Accordingly, a fraction of TLR9-cherry colocalizes with the lysosomal marker Lamp1-GFP in CpG-stimulated cells.

Supplementary Figure 3. TLR9 and UNC93B colocalize with CD63 in CpG-stimulated wild type cells.

(**a**) TLR9-GFP was coexpressed with CD63-cherry in either B6 or 3d BM-DCs. (**b**) Wild type and mutant (H412R) UNC93B-GFP were coexpressed with CD63-cherry in B6 and 3d BM-DCs, respectively. After stimulation with 1 μM CpG for 2 h, twenty live cells from each group were randomly chosen and imaged for both GFP and cherry signals. Colocalization of GFP and cherry signals was quantified by using the MetaMorph colocalization analysis tools. Mean ± SEM of Pearson's correlation coefficients are 0.56 ± 0.05 for TLR9 vs. CD63 in B6 cells, -0.43 ± 0.04 for TLR9 vs. CD63 in 3d cells, 0.43 ± 0.06 for wild type UNC93B vs. CD63 in wild type cells and -0.39 ± 0.03 for mutant UNC93B vs. CD63 in 3d cells. *; $P < 0.001$, *t*-test

Supplementary Figure 4. TLR7 fails to translocate to endolysosomes upon stimulation of 3d BM-DCs.

TLR7-YFP was coexpressed with CD63-cherry in either B6 or 3d BM-DCs and live cells were imaged after stimulation with 1 μM imiquimod for 2 h. In 3d cells, TLR7 remains in the ER, whereas it colocalizes with CD63 in endolysosomes of B6 cells.

Supplementary Figure 5. Localization of TLR4 is normal in 3d cells.

TLR4-YFP was expressed in either B6 or 3d BM-DCs and live cells were imaged after stimulation with 1 μg/ml LPS for 2 h. Shown are two exemplary BM-DCs from each mouse. In both B6 and 3d cells, TLR4 localizes to the cell surface and inside the cells.

Supplementary Figure 6. Localization of class I and class II MHC molecules is normal in 3d cells.

(**a**) BM-DCs from B6 and 3d mice were fixed, permeabilized and stained for MHC class I. In both wild type and 3d dendritic cells, Class I MHC is present at the cell surface and in the perinuclear region. (**b**)

BM-DCs from B6 and 3d mice were fixed, permeablized and stained for MHC class II (green) and Lamp1 (red). In both wild type and 3d dendritic cells, class II MHC molecules reside in Lamp1-postive endosomes. White dashed lines mark the cell boundary. (**c**) Wild type (red line) or 3d (blue line) BM-DCs were stimulated with 1 μg/ml LPS (left), 1 μM CpG (middle) or 1 μM imiquimod (right) for 16 h and the level of MHC class II on the cell surface was measured by staining with PE-conjugated anti-I-A^b antibody and flow cytometry. LPS upregulated the surface level of MHC class II equally in B6 and 3d cells. In contrast, CpG and imiquimod failed to increase the MHC class II level in 3d cells. (**d**) B6 or 3d BM-DCs were stimulated with 1 μg/ml LPS or 1 μM CpG for 16 h, fixed, permeabilized and stained for MHC class II and Lamp1. In both B6 and 3d cells, MHC class II molecules were mainly found at the cell surface upon LPS stimulation. Therefore, translocation of Class II MHC to the plasma membrane does not require UNC93B. When cells were stimulated with CpG, less MHC class II molecules were found at the cell surface of 3d cells. Scale bars; 10 μm

Supplementary Figure 7. Wild type but not mutant UNC93B translocates to endolysosomes in cells stimulated with CpG.

(**a**) Wild type UNC93B-GFP was coexpressed with cherry-KDEL in HEK-293T cells by transient transfection. Cells were imaged at 24 h post-transfection. UNC93B-GFP colocalizes with the ER marker cherry-KDEL. (**b**) Wild type UNC93B-cherry was expressed in BM-DCs from MHC class II-GFP knock-in mice in which the endogenous I-$A^b\beta$ molecule was substituted with GFP-tagged I-$A^b\beta$ [1]. Cells were imaged after stimulation with 1 μM CpG for 2 h. UNC93B colocalizes with MHC class II in endolysosomes. (**c**) BM-DCs from B6 mice were stimulated with 1 μM CpG for 2 h, fixed, permeabilized and stained with affinity purified rabbit polyclonal anti-UNC93B (green) and monoclonal Lamp1 (red) antibodies. Endogenous wild type UNC93B colocalizes with Lamp1. (**d**) Wild type and H412R mutant UNC93B-GFP proteins were coexpressed with CD63-cherry in B6 and 3d BM-DCs, respectively. Live cells were imaged after stimulation with 1 μM CpG for 2 h. Only wild type UNC93B colocalized with CD63 in endolysosomes. (**e**) Wild type or H412R mutant UNC93B-GFP proteins were stably coexpressed with cherry-KDEL in RAW macrophages. Live cells were imaged after stimulation with 1 μM CpG for 2 h. A large fraction of wild type UNC93B-GFP does not colocalize with cherry-KDEL, indicating that they are exported from the ER. In contrast, mutant UNC93B-GFP remained colocalized with cherry-KDEL.

Supplementary Figure 8. Signals that promote transport of UNC93B/TLR complex may originate from multiple receptors. Wild type but not mutant UNC93B binds to the nucleotide sensing TLRs in the ER and delivers them to endosomes. Signals that promote transport of UNC93B/TLR complex may originate from receptors that include the TLRs themselves, but possibly also receptors that remain to be identified. These receptors could be located at the surface, in endosomes or even in the ER. Blue arrows; Small amounts of agonist for TLR7 and TLR9 may reach the ER - via a route known to delivers some bacterial toxins and viruses[2] - and thus stimulate the receptors to induce transport of the remainder of the ER-resident TLRs to endosomes. Alternatively, a minor fraction of TLR7 and TLR9 may constitutively reside in endosomes and sense the internalized agonists. Activation of such TLRs leads to recruitment of the ER-resident TLRs to endosomes. Yellow arrows; Activation of TLR4 by LPS also results in translocation of UCN93B/TLR complexes from the ER to endosomes. Pink arrows; The as yet unidentified protein (depicted as a purple rod) may recognize TLR agonists and promote transport of UCN93B/TLR complexes to endosomes. In endosomes, TLR7 and TLR9 recognize the bulk of internalized agonists and initiate signaling via recruitment of MyD88 adaptor molecules.

Refereces

1. Boes, M. et al. T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* **418**, 983-8 (2002).
2. Spooner, R. A., Smith, D. C., Easton, A. J., Roberts, L. M. & Lord, J. M. Retrograde transport pathways utilised by viruses and protein toxins. *Virol J* **3**, 26 (2006).