At least two members of the Toll-like receptor (TLR) family, TLR7 and TLR9, can recognize self-RNA and self-DNA, respectively. Despite the structural and functional similarities between these receptors, their contributions to autoimmune diseases such as systemic lupus erythematosus can differ. For example, TLR7 and TLR9 have opposing effects in mouse models of systemic lupus erythematosus—disease is exacerbated in TLR9-deficient mice but attenuated in TLR7-deficient mice. However, the mechanisms of negative regulation that differentiate between TLR7 and TLR9 are unknown. Here we report a function for the TLR trafficking chaperone UNC93B1 that specifically limits signalling of TLR7, but not TLR9, and prevents TLR7-dependent autoimmunity in mice. Mutations in UNC93B1 that lead to enhanced TLR7 signalling also disrupt binding of UNC93B1 to syntenin-1, which has been implicated in the biogenesis of exosomes. Both UNC93B1 and TLR7 can be detected in exosomes, suggesting that recruitment of syntenin-1 by UNC93B1 facilitates the sorting of TLR7 into intraluminal vesicles of multivesicular bodies, which terminates signalling. Binding of syntenin-1 requires phosphorylation of UNC93B1 and provides a mechanism for dynamic regulation of TLR7 activation and signalling. Thus, UNC93B1 not only enables the proper trafficking of nucleic acid-sensing TLRs, but also sets the activation threshold of potentially self-reactive TLR7.

Mutations in UNC93B1 enhance TLR7 signalling

To uncover such mechanisms, we investigated UNC93B1, a trafficking chaperone that is required for TLRs to exit the endoplasmic reticulum (ER) and traffic to endosomes. The continued association of UNC93B1 with TLRs after leaving the ER suggested that this protein may mediate other regulatory steps. We performed a scanning-alanine mutagenesis screen of UNC93B1 in mouse RAW macrophage lines (Extended Data Fig. 1b). The screen identified several mutations—524-PRQ/AAA-526, 530-PKP/AAA-532, 545-DNS/AAA-547 and 548-DES/AAA-550—that enhanced TLR7 responses relative to cells expressing wild-type UNC93B1, without affecting TLR3 or TLR9 responses (Fig. 1a, b). Non-functional UNC93B1(H412R) served as negative control. These mutations were all within a 33-amino-acid region in the UNC93B1 C-terminal tail (residues 521–553) (Fig. 1c), which suggests that the phenotypes associated with these mutants may be linked by a common mechanism. UNC93B1PKPAAA (hereafter referred to as UNC93B1PKP) cells displayed enhanced activation of MAPKs (p38, JNK and ERK) as well as stronger degradation of IkBα (Extended Data Fig. 1a). Moreover, assembly of the myddosome complex, the most proximal signalling step downstream of TLR7 activation, was increased in mutant cells (Fig. 1d). This enhanced signalling was not due to differences in the expression or stability of the UNC93B1 mutants, as protein levels were similar among the RAW macrophage lines (Extended Data Fig. 1b).

A previously described mutation (D34A) near the N terminus of UNC93B1 enhances TLR7 signalling by increasing TLR7 export from the ER at the expense of TLR9. However, unlike UNC93B1(D34A), the trafficking of both TLR7 and TLR9 appeared normal in UNC93B1PKP-expressing cells, as shown by normal amounts of cleaved endosomal receptor (Extended Data Fig. 2a–c). Pulse-chase analysis of TLR7 showed that ectodomain cleavage of TLR7 occurred with normal kinetics in UNC93B1PKP cells (Extended Data Fig. 3a), and TLR7 levels were similar in phagosomes isolated from wild-type and PKP mutant UNC93B1 cells (Extended Data Fig. 3b), which suggests that TLR7 trafficking to endosomes is generally equivalent. We also considered the possibility that UNC93B1PKP alters TLR7 subcellular localization, thereby enhancing accessibility to ligand. However, colocalization between TLR7 and the late endosomal marker Lamp1 was similar in cells expressing wild-type UNC93B1 and UNC93B1PKP but much reduced in cells expressing non-functional UNC93B1(H412R) (Extended Data Fig. 3c).
Fig. 1 | Syntenin-1 binds to the C-terminal tail of UNC93B1 and restricts TLR7 signalling. a, Intracellular cytokine staining of TNF in mouse RAW macrophage lines expressing the indicated UNC93B1 alleles and stimulated with Cpg-B (100 nM), R848 (10 ng ml⁻¹), single-stranded RNA (ssRNA; 2.5 µg ml⁻¹), poly(I:C) (20 µg ml⁻¹) or LPS (10 ng ml⁻¹). Shaded histograms are unstimulated controls. HR, non-functional H412R mutant UNC93B1; WT, wild type. b, TNF production, measured by ELISA, from the indicated RAW macrophage lines after stimulation for 8 h with R848 (10 ng ml⁻¹), ssRNA (1 µg ml⁻¹), CpG-B (25 nM) or LPS (50 ng ml⁻¹). Data are mean ± s.d., n = 2 biological replicates. PKP, UNC93B1 mutant PKP/AAA. c, Topology of UNC93B1 with the C-terminal regulatory region indicated in orange. aa, amino acids. d, Immunoprecipitation (IP) of MyD88 from RAW macrophage lines expressing the indicated UNC93B1–Flag alleles and stimulated with R848 (500 ng ml⁻¹) followed by immunoblot for IRAK2. Input levels of MyD88 and IRAK2 in whole-cell lysates (WCL) are also shown. e, Silver stained SDS-PAGE gel of purified UNC93B1–Flag complexes from phagosomes of RAW macrophages expressing the indicated UNC93B1–Flag alleles. The 32-kDa protein corresponding to syntenin-1 is indicated. f, Purified UNC93B1–Flag complexes described in e were immunoblotted for syntenin-1. g, Syntenin-1 binding to UNC93B1 was measured by Flag immunoprecipitation followed by immunoblot for syntenin-1 from the indicated RAW macrophage lines stimulated with R848 (0.5 µg ml⁻¹). h, Interaction between syntenin-1 and UNC93B1 was measured as described in g from wild-type UNC93B1 RAW macrophages stimulated with R848 (0.5 µg ml⁻¹) or CpG-B (0.5 µM). i, NF-κB activation in HEK293T cells transiently expressing TLR7 and increasing amounts of syntenin-1 was measured using a dual luciferase reporter assay. Cells were stimulated with R848 (50 ng ml⁻¹) for 16 h before collection. RLUs, relative luciferase units, normalized to Renilla expression (n = 3 biological replicates). Vec, vector control. ***P < 0.0001, one-way ANOVA. All data are representative of at least three independent experiments.

Also, UNC93B1PKP itself colocalized normally with LAMP1 (Extended Data Fig. 3d). Together, these results suggest that the C-terminal tail of UNC93B1 specifically regulates TLR7 activation through a novel mechanism.

Syntenin-1 binds UNC93B1 and inhibits TLR7 signalling

To test whether UNC93B1 interferes with TLR7 signalling through association with an unknown negative regulator, we searched for proteins that interact with wild-type but not UNC93B1PKP. One challenging aspect of this approach is the relatively small fraction (<5%) of UNC93B1 in endosomes relative to the ER (Extended Data Figs. 3d, 4a). To overcome this obstacle, we enriched for the endosomal pool of UNC93B1 by first isolating phagosomes from RAW cells followed by purification of UNC93B1 protein complexes via anti-Flag antibodies (see scheme in Extended Data Fig. 4b). This approach revealed an approximately 32-kDa band present in wild-type UNC93B1 samples that was reduced in UNC93B1PKP samples (Fig. 1e). We used tandem mass spectrometry to identify this band as syntenin-1 (also known as syndecan binding protein, SDCBP), which we confirmed by immunoblot, using an anti-syntenin-1 monoclonal antibody (Fig. 1f).

Syntenin-1 is a PDZ domain-containing adaptor protein that can influence the trafficking of transmembrane proteins but has also been reported to regulate assembly of signalling complexes, including signalling downstream of TLRs. After stimulation of TLR7, the association of syntenin-1 with wild-type UNC93B1 was rapidly and transiently increased, whereas the interaction with UNC93B1PKP did not increase (Fig. 1g). In line with UNC93B1PKP having no effect on TLR9 or TLR3 signalling, there was no increased association between UNC93B1 and syntenin-1 after stimulation with Cpg-B or polyinosinic:polycytidylic acid (poly(I:C)), respectively (Fig. 1h and Extended Data Fig. 5a). Similarly, syntenin-1 was coimmunoprecipitated with TLR7 but not with TLR9 (Extended Data Fig. 5b). A previous study reported that overexpression of syntenin-1 can inhibit TLR4 and IL-1 receptor signalling (IL-1R) signalling by interfering with the interaction between IRAK-1 and TRAF6. We observed that overexpression of syntenin-1 also inhibited TLR7 signalling (Fig. 1i), whereas TNFR signalling was not as affected (Extended Data Fig. 5c). Similar results were obtained with the closely related syntenin-2 (also known as SDCBP2) protein (Extended Data Fig. 5d). However, we could not reproduce the reported interaction between syntenin-1 and IRAK-1 or TRAF6 (data not shown).

TLR7–UNC93B1 complexes are sorted into MVBs

Syntenin-1 has also been implicated in the ESCRT-mediated uptake of specific transmembrane receptors into intraluminal vesicles (ILVs) of multivesicular bodies (MVBs). This process can terminate receptor signalling, so we considered whether this mechanism could explain
Fig. 2 | UNC93B1–TLR7 complexes are sorted into ILVs of MVBs. a, Exosome preparations were enriched for the exosome markers CD63, ALIX and syntenin-1, and devoid of the ER marker calnexin. Whole-cell lysates or lysates of exosomes from RAW macrophages expressing wild-type UNC93B1 or non-functional H412R mutant (HR) were probed with antibodies against the indicated proteins. Equivalent amounts of total protein were loaded per lane. b, Sorting of TLR7 into exosomes of RAW macrophages requires UNC93B1. Immunoblot for TLR7–HA and UNC93B1–Flag of exosome preparations from RAW macrophages expressing wild-type UNC93B1 or the HR non-functional UNC93B1 mutant. Samples were normalized to CD63 levels to ensure equivalent loading. c, UNC93B1–Flag was immunoprecipitated from lysates of the indicated RAW macrophage lines and total or K63-linked ubiquitylation (Ub) was measured by immunoblot. Arrowheads indicate the mobility of the indicated RAW macrophage lines and total or K63-linked ubiquitylation indicated RAW macrophage lines after stimulation for 8 h with R848 (10 ng ml\(^{-1}\)) for TLR7–HA and UNC93B1–Flag of exosome preparations from RAW macrophages. d, Schematic showing the relative positions of lysine residues analysed in c and e. e, TNF production, measured by ELISA, from the indicated RAW macrophage lines after stimulation for 8 h with R848 (10 ng ml\(^{-1}\)) or LPS (10 ng ml\(^{-1}\)). Data are mean ± s.d. of \(n = 3\) (\(n = 2\) for K197R and HR) biological replicates, representative of two independent experiments. *P value determined by unpaired two-tailed Student’s t-test. **P value determined by unpaired two-tailed Student’s t-test.

Fig. 3 | Serine phosphorylation in the C-terminal tail of UNC93B1 regulates syntenin-1 recruitment. a, TNF production, measured by ELISA, from the indicated RAW macrophage lines after stimulation for 8 h with R848 (20 ng ml\(^{-1}\)). Data are mean ± s.d., \(n = 3\) biological replicates. **P = 0.0002 for PKP compared to WT, ***P < 0.0001 for all other mutants compared to WT, unpaired two-tailed Student’s t-test. b, Syntenin-1 binding to wild-type or S547A/S550A mutant UNC93B1 in RAW macrophages stimulated with R848 (0.5 µg ml\(^{-1}\)) was measured by UNC93B1–Flag immunoprecipitation followed by immunoblot for syntenin-1. c, UNC93B1–Flag was immunoprecipitated from lysates of the indicated RAW macrophage lines and phosphorylation (P) of Ser547 and Ser550 was measured by immunoblot with phospho-specific UNC93B1 antibodies. Each blot was performed on the same membrane but cropped to present relevant lanes. d, RAW macrophages were stimulated with R848 (0.5 µg ml\(^{-1}\)) and CpG-B (0.5 µM) and UNC93B1 phosphorylation was measured by immunoblot after UNC93B1–Flag immunoprecipitation. e, A model of syntenin-1 recruitment to UNC93B1. All data are representative of at least three independent experiments.

how UNC93B1 dampens TLR7 signalling. To probe ILV contents for TLR7 and UNC93B1, we purified exosomes from the supernatants of RAW cells. Exosome preparations were enriched for classic ILV markers, such as CD63, ALIX and syntenin-1, and lacked markers of other organelles (Fig. 2a). TLR7 and UNC93B1 were present in exosomes from wild-type UNC93B1 cells, but absent in exosomes isolated from UNC93B1(H412R)-expressing cells (Fig. 2b). These results indicate that TLR7 is loaded into ILVs and that this loading requires functional UNC93B1.

K63-linked ubiquitylation often marks cargo for sorting into ILVs\(^{25}\), accordingly, immunoprecipitation of UNC93B1 from RAW cells followed by immunoblotting for ubiquitin revealed K63 ubiquitylation (Fig. 2c). Ubiquitylation of UNC93B1\(^{\text{PKP}}\) was substantially reduced, consistent with ubiquitylation and syntenin-1 recruitment both being required for sorting into ILVs. We individually mutated five cytosolic lysine residues to arginine (Fig. 2d) and identified Lys333 as necessary for much, but not all, of the ubiquitylation of UNC93B1 (Fig. 2c). The reduced ubiquitylation of UNC93B1(K333R) correlated with enhanced turnover of TLR7 into ILVs masks the relatively small pool of activated receptors that are sorted in a syntenin-mediated manner after stimulation. Indeed, the number of TLR molecules that engage ligand and become activated has previously been shown to be small\(^{26}\), and it is known that even small increases in TLR7 levels are sufficient to affect self versus non-self discrimination\(^{10,13}\).

Phospho-UNC93B1 regulates syntenin-1 recruitment

To investigate the basis of syntenin-1 recruitment to UNC93B1, we focused on additional post-translational modifications within the UNC93B1 C-terminal tail. Global phosphoproteomic analyses have identified Ser547 and Ser550 as potential UNC93B1 phosphorylation sites (https://www.phosphosite.org/proteinAction.action?id=14824), and alanine mutations in our original screen that included these serine residues (DNS/AAA and DES/AAA) lead to enhanced TLR7 responses (Fig. 1a). Single mutations of UNC93B1 residues Ser547 (S547A), Ser550 (S550A), or both serine residues (S547A/S550A) were sufficient to enhance TLR7 responses to levels comparable to UNC93B1\(^{\text{PKP}}\) without affecting other TLRs (Fig. 3a and Extended Data Fig. 7c–e). Using phospho-specific UNC93B1 antibodies raised against Ser547 and Ser550 (Extended Data Fig. 7a), we confirmed that UNC93B1 is indeed phosphorylated at these residues in RAW cells; mutation of either serine reduced detection by the phospho-specific antibody, whereas mutation of both serine residues completely abrogated detection (Extended Data Fig. 7b). Combining the UNC93B1\(^{\text{PKP}}\) mutation with the UNC93B1 serine mutations S547A, S550A or S547A/S550A did not further enhance TLR7 responses (Extended Data Fig. 7c), which suggests that each mutation
acts by disrupting the same mechanism. Syntenin-1 recruitment to UNC93B1 after R848 stimulation was impaired in UNC93B1(S547A/S550A)-expressing cells (Fig. 3b). To determine whether the failure of UNC93B1PKP to recruit syntenin-1 results from absent phosphorylation, we examined the phosphorylation of Ser547 and Ser550 in UNC93B1PKP-expressing cells (Fig. 3c). These results support a model in which syntenin-1 binding to UNC93B1 requires specific residues within the C-terminal tail as well as phosphorylation of Ser547 and Ser550 (Fig. 3e). Although some syntenin-1 is associated with UNC93B1 in unstimulated cells (consistent with the basal levels of UNC93B1 phosphorylation in resting cells), the interaction is further increased after TLR7 signalling. Thus, the mechanism we describe not only appears to influence the initial threshold of TLR7 activation but also operates as a negative feedback loop to shut down TLR7 signalling by sorting UNC93B1 complexes into ILVs. Defining the signals that lead to this increased recruitment, including the identities of the kinases and phosphatases regulating phosphorylation of UNC93B1, will be an important aspect of future work.

**SNPs in the UNC93B1 C-tail enhance TLR7 responses**

To determine whether UNC93B1 and syntenin-1 regulation of TLR7 could be relevant in humans, we searched publicly available human genomic data for single nucleotide polymorphisms (SNPs) within the UNC93B1 C-terminal tail, which is highly conserved between mouse and human. Four very rare (minor allele frequencies of 0.04% or lower, according to the 1000 Genomes project) coding variants within this region have been reported: P352T, Y359D, D545V and D545Y. Three of the UNC93B1 variants (Y359D, D545V and D545Y) increased TLR7 responses relative to wild-type UNC93B1 in human HEK293T cells although UNC93B1(Y359D), and to a lesser extent UNC93B1(D545Y), also slightly increased TLR5 responses (Extended Data Fig. 8). These alleles are too rare to be linked to autoimmune disorders via

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**Fig. 4** UNC93B1PKP knock-in mice develop TLR7-driven systemic inflammation and autoimmunity. **a.** Gross appearance and weights of UNC93B1WT/PKP mice compared to littermate controls. **b.** Flow cytometric analysis of the indicated immune cell populations in spleen or lymph nodes in Unc93b1WT/PKP, Unc93b1PKP/PKP and Unc93b1PKP/PKP mice at 6–8 weeks of age. Data points were pooled from four independent experiments. **c.** Genotypes and allele frequencies for single nucleotide polymorphisms in the UNC93B1 C-tail. **d.** Enhanced response to TLR7 stimulation was observed in BMDCs derived from indicated TLR7–/– mice. **e.** Gross appearance and weights of 6-week-old mice with indicated genotypes are shown. Data were pooled from three independent experiments. **f.** Proposed model of syntenin-1-mediated restriction of TLR7 signalling. After activation of TLR7, binding of syntenin-1 to UNC93B1 may facilitate sorting of the TLR7–UNC93B1 complex into ILVs of MVBS, which terminates TLR7 signalling. Disruption of syntenin-1 binding (for example, in UNC93B1PKP–expressing cells) prevents the inducible sorting of TLR7–UNC93B1 into MVBS, leading to unrestrained signalling and TLR7-driven autoimmunity. Additional mechanisms responsible for the steady-state turnover of TLR7 and/or UNC93B1 are not depicted. ESCRT-III, endosomal sorting complexes required for transport III. All P values determined by unpaired two-tailed Student’s t test.
UNC93B1(PKP) mice develop autoimmunity

Finally, we sought to test the importance of UNC93B1 and syntenin-1 regulation of TLR7 the discrimination of self and non-self in vivo. We reasoned that analysis of syntenin-1-deficient mice could be complicated for several reasons. First, syntenin-1 has been implicated in the regulation of multiple transmembrane proteins via its role in MVB biogenesis\(^2^2\). Although syntenin-1-deficient mice show pleiotropic effects on the immune system and the microbiota\(^2^7\), syntenin-2 probably compensates for syntenin-1 deficiency. Indeed, our attempts to generate cells lacking both syntenin-1 and syntenin-2 suggest that the mutant cells experience severe fitness defects (data not shown). To circumvent these issues, we introduced the UNC93B1\(^{PKP}\) mutation into the germline of mice using Cas9 genome editing (Extended Data Fig. 9a). This mutation disrupts interaction between syntenin-1 and UNC93B1 and should leave other syntenin-1 functions unaffected. Unc93b1\(^{PKP/PKP}\) mice were born below the expected Mendelian frequency and were severely runted (Fig. 4a). These mice exhibited hallmarks of systemic inflammation and autoimmunity previously described in TLR7-overexpressing mice\(^2^3\), including increased frequencies of activated T cells, loss of marginal zone B cells, increased frequencies of MHC\(^*\) dendritic cells and inflammatory monocytes in secondary lymphoid organs, and evidence of emergency granulopoiesis (Fig. 4b and Extended Data Fig. 9b, c). Unc93b1\(^{PKP/PKP}\) mice developed anti-nuclear antibodies very early in life (Fig. 4c and Extended Data Fig. 9d). Unc93b1\(^{PKP/PKP}\) mice also showed signs of immune dysregulation but not to the same extent as Unc93b1\(^{WT/WT}\) mice (Fig. 4b–d and Extended Data Fig. 9b–g).

Bone marrow-derived dendritic cells (BMDCs), macrophages and B cells from Unc93b1\(^{WT/PKP}\) and Unc93b1\(^{PKP/PKP}\) mice mounted stronger responses to TLR7 ligands than Unc93b1\(^{WT/WT}\) cells, whereas responses to TLR9 and TLR4 ligands were equivalent (Fig. 4d and Extended Data Fig. 9e–i). In line with the enhanced cytokine production, macrophages from Unc93b1\(^{PKP/PKP}\) mice showed stronger assembly of the mydosome complex downstream of TLR7 activation (Extended Data Fig. 9f). These enhanced TLR7 responses were not due to differences in UNC93B1 expression, as UNC93B1 protein levels in bone marrow-derived macrophages (BMMs) were similar across all genotypes. (Extended Data Fig. 9k). Consistent with TLR7-driven disease, a lack of TLR7 completely rescued disease in Unc93b1\(^{WT/PKP}\) mice (Fig. 4c, e).

Together, our findings identify a regulatory mechanism of UNC93B1 that specifically limits TLR7 signalling and prevents TLR7-driven autoimmunity. We propose a model in which the C-terminal tail of UNC93B1 binds the adaptor protein syntenin-1, which facilitates sorting of recently activated TLR7 into IISVs of MVBs for protein turnover and/or sequestration and limits signalling in response to self-RNA (Fig. 4f). It is likely that other mechanisms can contribute to turnover of endosomal TLRs. In an accompanying paper, we demonstrate that TLR9 and TLR3, unlike TLR7, are released from UNC93B1 within endosomes\(^7\), which rules out the involvement of UNC93B1 in directly regulating degradation of these TLRs. This release mechanism may explain why UNC93B1 mutations that disrupt syntenin-1 binding, as well as UNC93B1 ubiquitylation or phosphorylation, do not affect TLR9 or TLR3 signalling, which is particularly interesting when considering the differential roles played by TLR7 and TLR9 in mouse models of systemic lupus erythematosus\(^1\). Even more intriguing is the possibility that recruitment of syntenin-1 to UNC93B1 can be dynamically controlled by phosphorylation. Identifying the factors involved in this regulation should reveal crucial determinants that influence the discrimination of self versus non-self and the development of autoimmunity.
Methods

 Antibodies and reagents

The following antibodies were used for immunblots and immuno-precipitations: anti-hA as purified antibody or matrix (3F10, Roche), anti-Flag as purified antibody or matrix (M2, Sigma-Aldrich), anti-mouse LAMP1 (AF4320, R&D Systems), anti-calanxin (ADI-SPA-860, Enzo Life Sciences), anti-GAPDH (GT239, GenTex), anti-MyD88 (AF3109, R&D Systems), anti-IRAK2 (Cell Signaling), anti-Phospho-p38 (Cell Signaling), anti-p38 (Cell Signaling), anti-Phospho-SAPK/JNK (81E1, Cell Signaling), anti-SAPK/JNK (56G8, CellSignaling), anti-Phospho-p44/42 ERK1/2 (D13.14.4E, Cell Signaling), anti-p44/42 ERK1/2 (137F5, Cell Signaling), anti-IkBa (Cell Signaling), anti-syntenin-1 (2C12, Novusbio), anti-UNC93B1 (PAS-20510, Thermo Scientific), anti-ubiquitin (P4D1, Santa Cruz), anti-K63-linked ubiquitin (human polyclonal, gift from M. Rape), goat anti-mouse IgG-AlexaFluo680 (Invitrogen), goat anti-mouse IgG-AlexaFluo680 (Invitrogen), rabbit anti-goat IgG-AlexaFluo680 (Invitrogen), goat anti-human IRDye 680RD (Lico), goat anti-mouse IRDye 800CW (Lico), donkey anti-rabbit IRDye 680RD (Lico), goat anti-rat IRDye 800CW (Lico). Antibodies for immunofluorescence were: rat anti-hA (3F10, Roche), rabbit anti-lAMP1 (ab24170, Abcam), goat anti-rat IgG-AlexFluo488 (Jackson ImmunoResearch), goat anti-rabbit IgG-AlexFluo647 (Jackson ImmunoResearch), cells were mounted in Vectashield Hard Set Mounting Medium for Fluorescence (Vector Laboratories). For ELISA: mouse TNF purified (IF3F3D4, Bioscience), anti-mouse TNF-biotin (XT3/XT22, Bioscience), streptavidin-HRP (BD Pharmingen). Antibodies and reagents used for flow cytometry were: anti-TNF (MP6-TX22, bioscience), purified anti-CD16/32 Fc Block (2.4G2), CD3e (145-2C11, Biologend), CD4 (GK1.5, Biologend), CD8 (53-6.7, BioLegend), CD11b (M1/70, BioLegend), Ly6G (1A8, TONBO biosciences), Ly6C (HK1.4, Biologend), F4/80 (CI:A3-1, AbD serotec), MHCII (M5/114.15.2, BioLegend), CD64 (MEL-14, bioscience), CD69 (H1.2F3, Bioscience), CD1d (1B1, Bioscience), CD220 (RA-3-6b2, Invitrogen), CD19 (6D5, Biologend), IgD (11-26.2a, Biologend), IgM (eB12-15F9, bioscience), CD21 (eB8D9, bioscience), CD23 (B3B4, Bioscience), CD138 (281-2, Biologend), CD11b (MI/70, Biologend), Ly6G (IA8, TONBO biosciences), Ly6C (HK1.4, Biologend), F4/80 (CI:A3-1, AbD serotec), MHCII (M5/114.15.2, Biologend), C11c (N418, Biologend), CD17 (c-Kit) (28B, bioscience), SCA-1 (D7, bioscience). For anti-nuclear antibody detection: anti-mouse IgG-AlexFluo 488 (Jackson Immuno-research), anti-mouse IgM-FITC (Invitrogen).

The antibody against phosphorylated UNC93B1 was generated by Invitrogen against phosphorylated peptide (YLED(NpS)DE(ps)DMEGEQ) using their ‘Rabbit, 90-Day immunization’ protocol. Antibody in sera was enriched with immobilized phospho-peptide, followed by negative absorption with unphosphorylated peptide.

CpG-B (ODN1668: TCCATGACGTTCCTGATGCT, all phosphorothioate linkages) was synthesized by Integrated DNA Technologies. R848, poly(I:C) HMW, ssRNA40/LyoVec and LPS were purchased from Invivo-Gen. Human IL-1β was from Invitrogen. NP-40 (Igepal CA-630) was from Sigma-Aldrich. Lipofectamine-2000 reagent (Invitrogen) and OptiMEM-1 (Invitrogen) were used for transfection of plasmid DNA. ProMag1 Series-COOH Surfactant free magnetic beads (25029) for phagosome preparations were purchased from Polysciences. For luciferase assays: Renilla substrate: coelenterazine native (Biotium), firefly substrate: luciferin (Biosyn), Passive Lysis Buffer, 5x (Promega).

Cells and tissue culture conditions

HEK293T (from ATCC) and GP2-293 packaging cell lines (Clontech) were cultured in DMEM complete medium supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine, penicillin–streptomycin, sodium pyruvate and HEPES (pH 7.2–7.5) (Gibco). RAW264 macrophage cell lines (ATCC) were cultured in RPMI1640 (same supplements as above). BMMs were differentiated for 7 days in RPMI complete medium (same supplements as above plus 0.00034% (v/v) β-mercaptoethanol) and supplemented with 10% (v/v) M-CSF containing supernatant from 3T3-CSF cells. BMDCs were differentiated for 7 days in RPMI complete medium (same supplements as above plus 0.00034% (v/v) β-mercaptoethanol) and supplemented with 2% (v/v) GM-CSF containing supernatant from J558L cells.

To generate HEK293T UNC93B1– cells, guide RNAs were designed and synthesized as gblocks as previously described28 and then were subcloned into pUC19 (guide RNA: CTACACTACGGCGGTTCATT). Humanized Cas9-2xNLS-GFP was a gift from the Doudna laboratory, University of California, Berkeley. HEK293T cells were transfected using Lipofectamine LTX with equal amounts of the guide RNA plasmid and Cas9 plasmid. Seven days after transfection, cells were plated in a limiting-dilution to obtain single cells. Correct targeting was verified
Article

by PCR analysis and loss of response to TLR9 and TLR7 stimulation in an NF-κB luciferase assay. UNC93B1<sup>−/−</sup> RAW macrophages were generated with the Cas9(D10A)-GFP nickase (guide RNAs: (1) GCGCTGTCGGCCG TAGTACGG; (2) CGAGTGTGAACGCTGTCGG; (3) TCTGGATGC GGGCTGTCGGCCG; and (4) AGTCGGCGCTACGTCAGTGG). Macrophages were transduced with Cas9(D10A) and all four guide RNAs using Lipofectamine LTX and Plus reagent and single cell-sorted on Cas9–GFP two days later. Correct targeting was verified by loss of response to TLR7 stimulation and sequencing of the targeted clones after TOPO cloning. MyD88 was knocked out in Unc93B1<sup>−/−</sup> RAW macrophages stably expressing TLR7–HA and either wild-type UNC93B1 or UNC93B1<sup>ΔN</sup>. Cas9 transfection and screening of cells was performed as before, except usingCas9-2xNLS-GFP (guide RNA: GGTTCAGACACA GCCATAGG).

Retrospective transduction

Retrospective transduction of RAW macrophages was performed as previously described<sup>46</sup>. For macrophages expressing the UNC93B1 mutant library, transduced cells were selected with puromycin starting 48 h after transduction and the efficiency of drug selection was verified by equal mCherry expression of target cells. When necessary, target cells were sorted on a Becton Dickinson Aria Fusion Sorter to match UNC93B1 expression levels using the bicistronic fluorescent reporter. For retroviral transduction of BMMs, bone marrow was collected and cultured in M-CSF-containing RPMI for two days. Progenitor cells were transduced with viral supernatant (produced as above) on two successive days by spinfection for 90 min at 32 °C. Forty-eight hours after the second transduction cells were put on puromycin selection and cultured in M-CSF-containing RPMI medium until collection on day 8.

Pulse-chase

Cells were seeded into 6 cm dishes the day before. After washing in PBS, cells were starved for 1 h in cysteine/methionine-free medium (Corning) containing 10% dialysed serum (dialysed in PBS for two days using a 10 kDa SnakeSkin), then pulsed with 0.25 mM Cys, 135 mM-cysteine/methionine (EasyTag Express Proteilabelling Mix, Perkin Elmer). After a 45 min pulse, cells were washed and cultured in 5 ml chase medium containing 0.45 mM l-cysteine and l-methionine or collected as the zero time point. Time points were collected as follows: cells were washed twice in 2 ml PBS, then scraped in PBS and cell pellets were subjected to haemagglutinin immunoprecipitation.

Cell fractionation by sucrose density-centrifugation

Cells in four confluent 15-cm dishes were washed in ice-cold PBS, scraped in 10 ml sucrose homogenization buffer (SHB; 250 µM sucrose, 3 mM imidazole, pH 7.4) and pelleted by centrifugation. Cells were resuspended in 2 ml SHB plus protease inhibitor cocktail with EDTA (Roche) and 1 mM PMSF and disrupted by 25 strokes in a steel dounce homogenizer. The disrupted cells were centrifuged for 10 min at 1,000g to remove nuclei. Supernatants were loaded onto continuous sucrose gradients (percentage iodixanol: 0, 10, 20, 30) and ultracentrifuged in an SW41 rotor at 113,832 g for 2 h (Optima L-90K Ultracentrifuge, Beckman Coulter). Twenty-two fractions of 420 µl were collected from top to bottom and 100 µl of each fraction were denatured in SDS buffer for western blot analysis. For immunoprecipitations, three fractions corresponding to ER or endosomes were combined and lysed for 1 h after addition of protease inhibitor cocktail and NP-40 to a final concentration of 1%. Coimmunoprecipitation with anti-HA matrix was performed as described below.

Exosome purification

Exosomes were purified as previously described<sup>46</sup>. In brief, RAW macrophages were grown in 4 × 15-cm dishes and 24 h before exosome collection, the cell culture medium was replaced with exosome-depleted medium (RPMI 1640, 10% FCS and supplements ultra-centrifuged overnight at 100,000g). The next day, cell supernatants were obtained, pooled (50 ml total) and subjected to sequential centrifugation steps at 4 °C: (1) 10 min at 300g to remove live cells; (2) 20 min at 2,000g to remove dead cells; (3) 30 min at 10,000g to remove debris; and (4) 70 min at 100,000g to pellet exosomes. Spins 3 and 4 were performed in an Optima L-90K Ultracentrifuge (Beckman Coulter) using an SW41 swinging-bucket rotor and 12 ml sample tubes. Exosomes were washed in PBS and centrifuged for another 60 min at 100,000g. Final exosome pellets were lysed in 50-70 µl PBS plus 1% NP-40 and Roche complete protease inhibitor cocktail for 30 min and then denatured in SDS loading buffer at room temperature for 1 h. For comparison of exosome protein contents to whole-cell lysates, some cells from the initial culture plates were lysed in NP-40 buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 5 mM EDTA, supplemented with Roche complete protease inhibitor cocktail) for 1 h at 4 °C, centrifuged at maximum speed for 30 min at 4 °C and then denatured in SDS loading buffer at room temperature for 1 h. Twenty microcentres of cell and exosome lysates were kept for protein quantification with the Micro BCA Protein Assay Kit (Thermo Fisher). Between 5 and 10 µg of total protein was loaded per lane for western blot analysis.

Luciferase assays

Activation of NF-κB in HEK293T cells was performed as previously described<sup>7</sup>. In brief, transfections were performed in OptiMEM-I (Invitrogen) with LTX transfection reagent (Invitrogen) according to manufacturer’s guidelines. Cells were stimulated with CpG-B (200 nM–1 µM), R848 (100–200 ng ml<sup>−1</sup>), or human IL-1β (20 ng ml<sup>−1</sup>) after 24 h and lysed by passive lysis after an additional 12–16 h. Luciferase activity was measured on an LMaxII-384 luminometer (Molecular Devices).

Immunoprecipitation, western blot and dot blot

Cells were lysed in NP-40 buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, supplemented with 1 mM PMSF, Roche complete protease inhibitor cocktail and PhosSTOP tablets). For ubiquitin blots, 40 mM N-ethylmaleimide (Sigma) was added to the lysis buffer. After incubation at 4 °C for 1 h, lysates were cleared of insoluble material by centrifugation. For immunoprecipitations, lysates were incubated with anti-HA matrix or anti-Flag matrix (both pre-blocked with 1% BSA-PBS) for at least 2 h and washed four times in lysis buffer. Precipitated proteins were eluted in lysis buffer containing 200 ng ml<sup>−1</sup> haemagglutinin or 3× Flag peptide, or denatured in SDS loading buffer at room temperature for 1 h. Proteins were separated by SDS–PAGE (Bio-Rad TGX precast gels) and transferred to Immobilon PVDF membranes (Millipore) in a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked with Odyssey blocking buffer, probed with the indicated antibodies and developed using the Licor Odyssey Blot Imager. For dot blot assays, diluted peptides were dropwise added to nitrocellulose blotting membranes (GE Healthcare). Membranes were dried at room temperature, blocked and probed using the Licor Odyssey blot system.

Cell lysis and co-immunoprecipitations for myddosome analyses were performed in the following buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40 and supplemented with EDTA-free complete protease inhibitor cocktail (Roche), PhosSTOP (Roche) and 1 mM PMSF. Lysates were incubated overnight with anti-MyD88 antibody at 4 °C and then Protein G agarose (pre-blocked with 1% BSA-PBS) was added for an additional 2 h. Beads were washed four times in lysis buffer, incubated in SDS loading buffer at room temperature for 1 h, separated by SDS–PAGE, and probed with the indicated antibodies.

Tissue collection

Spleens and lymph nodes were digested with collagenase XI and DNase I for 30 min and single-cell suspensions were generated by mechanical disruption. Red blood cells were lysed in ACK Lysing Buffer (Gibco).
Flow cytometry
Cells were seeded into non-treated tissue culture 24-well plates or round-bottom 96-well plates. The next day, cells were stimulated with the indicated TLR ligands. To measure TNF production, brefeldinA (BD GolgiPlug, BD Biosciences) was added to cells 30 min after stimulation, and cells were collected after an additional 5.5 h. Dead cells were excluded using a fixable live/dead stain (Violet fluorescent reactive dye, Invitrogen). Cells were stained for intracellular TNF with a Fixation & Permeabilization kit according to manufacturer’s instructions (eBioscience).

For flow cytometry on mouse cells, dead cells were excluded using a fixable live/dead stain (Aqua fluorescent reactive dye, Invitrogen) or DAPI and all stains were carried out in PBS containing 1% BSA (w/v) and 0.1% azide (w/v) including anti-CD16/32 blocking antibody. Cells were stained for 20 min at 4 °C with surface antibodies. Data were acquired on a LSRFortessa or X20 analyser (BD Biosciences). See Extended Data Fig. 10 for gating strategies.

ELISA and cytometric bead array
Cells were seeded at 10^4 cells per well into tissue culture-treated flat-bottom 96-well plates. The next day the cells were stimulated with the indicated TLR ligands. For TNF ELISAs, NUNC MaxiSorp plates were coated with anti-TNF at 1.5 µg ml⁻¹ overnight at 4 °C. Plates were then blocked with PBS and 1% BSA (w/v) at 37 °C for 1 h before cell supernatants diluted in PBS and 1% BSA (w/v) were added and incubated at room temperature for 2 h. Secondary anti-TNF–biotin was used at 1 µg ml⁻¹ followed by streptavidin–HRP. Plates were developed with 1 mg ml⁻¹ temperature for 2 h. Secondary anti-TNF–biotin was used at 1 µg ml⁻¹ and peptides were stained for 20 min at 4 °C with surface antibodies. Data were acquired on a LSRFortessa or X20 analyser (BD Biosciences). See Extended Data Fig. 10 for gating strategies.

Type I interferon production by BMDCs
BMDCs were seeded at 10^4 cells per well into tissue culture-treated flat-bottom 96-well plates. The next day the cells were stimulated with the indicated TLR ligands for 16 h. The next day, supernatants were collected in 96-well plates and analyzed using the Mouse Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions.

B cell proliferation assay
Spleens were digested with collagenase 8 (Sigma) and DNase-I for 45 min and red blood cells were lysed using ACK buffer (Gibco). Splenocytes were labelled with 12.5 µg ml⁻¹ carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37 °C and immediately underlaid with 3 ml FCS to spin out CFSE. Cells were taken up in RPMI medium (containing 10% FCS, 1-glutamine, penicillin–streptomycin, HEPES, sodium pyruvate and β-mercaptoethanol), counted and seeded at 200,000 cells per well in round-bottom 96-well plates. Cells were incubated in medium with various concentrations of CpG-B, R848 and luciferase activity was measured on an LMaxII-384 luminometer (Molecular Devices).

Phagosome isolation and protein complex purification
Cells in a confluent 15-cm dish were incubated with approximately 10^8 1 µm magnetic beads (Polysciences) for 4 h. After rigorous washing in PBS, cells were scraped into 10 ml SHB and pelleted by centrifugation. Cells were resuspended in 2 ml SHB plus protease inhibitor cocktail with EDTA (Roche) and 1 mM PMSF and disrupted by 25 strokes in a steel dounce homogenizer. The disrupted cells were gently rocked for 10 min on ice to free endosomes. Beads were collected with a magnet (Dynal) and washed four times with SHB plus protease inhibitor. After the wash, the final wash, phagosome preparations were denatured in 2× SDS buffer at room temperature for 1 h and analysed by western blot.

For protein complex purification, phagosome preparations were lysed in NP-40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, supplemented with 1 mM PMSF, complete protease inhibitor cocktail and PhosSTOP tablets (Roche)) on ice for 1 h. Magnetic beads were removed by magnet and insoluble components were precipitated by 15,000g spin for 20 min. Lysate was incubated with anti-Flag matrix for 3 h, followed by four washes in lysis buffer. Proteins were eluted in NP-40 buffer containing 200 ng ml⁻¹ 3× Flag peptide and were further applied to western blot. silver stain or trypsin in-solution digest for mass spectrometry.

Mass spectrometry
Proteins were simultaneously extracted from a gel slice and digested with trypsin, and the resulting peptides were dried and resuspended in buffer A (5% acetonitrile, 0.02% heptafluorobutyric acid). A nano liquid chromatography column that consisted of 10 cm of Polaris c18 5 µm packing material (Varian) was packed in a 100 µm inner diameter glass capillary with an emitter tip. After sample loading and washed extensively with buffer A, the column was then directly coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL...
linear ion trap mass spectrometer. An Agilent 1200 HPLC equipped with a split line so as to deliver a flow rate of 300 nl min⁻¹ was used for chromatography. Peptides were eluted using a min gradient from buffer A to 60% buffer B (80% acetonitrile, 0.02% heptafluorobutyric acid).

Protein identification and quantification were done with Integrated-Proteomics Pipeline (IP2, Integrated Proteomics Applications) using ProLuCID/Sequest, DTASelect2 and Census. Tandem mass spectra were extracted from raw files using RawExtractor and were searched against the mouse protein database (obtained from UNIPROT) plus sequences of common contaminants, concatenated to a decoy database in which the sequence for each entry in the original database was reversed. LTQ data was searched with 3000.0 milli-amu precursor tolerance and the fragment ions were restricted to a 600.0 p.p.m. tolerance. All searches were parallelized and searched on the VJC proteomics cluster. Search space included all fully tryptic peptide candidates with no missed cleavage restrictions. Carbamidomethylation (+57.02146) of cysteine was considered a static modification. We required 1 peptide per protein and both tryptic termini for each peptide identification. The ProLuCID search results were assembled and filtered using the DTASelect program with a peptide false discovery rate of 0.001 for single peptides and a peptide false discovery rate of 0.005 for additional peptides for the same protein. Under such filtering conditions, the estimated false discovery rate was zero for the data sets used.

Quantification and statistical analysis
Statistical parameters, including the exact value of \( n \) and statistical significance, are reported in the figures and legends, in which \( n \) refers to the number of repeats within the same experiment. Representative images have been repeated at least three times, unless otherwise stated in the figure legends. Data are judged to be statistically significant when \( P < 0.05 \) by Student’s \( t \)-test. To compare the means of several independent groups, a one-way ANOVA followed by a Tukey’s posttest was used. To compare means of different groups across a dose response, a two-way ANOVA followed by a Bonferroni posttest was used. *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \). Statistical analysis was performed in GraphPad PRISM 7 (GraphPad Software Inc.). No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment, except for the analysis of anti-nuclear antibody staining of serum samples.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
The data that support the findings of this study are either included within the manuscript or are available from the corresponding author on reasonable request. Source Data for Figs. 1–4 and Extended Data Figs. 1–9 are included in the online version of the paper. Gel source data can be found in Supplementary Fig. 1.

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Author contributions
O.M., B.L. and G.M.B designed experiments. O.M. and B.L. performed experiments and analysed the data for all figures. L.S.M.K. performed B cell stimulations. N.K. assisted with design of mass spectrometry experiments. G.M.B. wrote the manuscript. O.M., B.L. and G.M.B revised and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to B.L. or G.M.B.

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Extended Data Fig. 1 | A C-terminal region in UNC93B1 regulates TLR7 responses. a, UNC93B1<sup>PKP</sup>-expressing macrophages show enhanced TLR7 signalling. Immunoblot of phosphorylated (P)-p38, P-JNK, P-ERK and IκBα of RAW macrophages stimulated with R848 (50 ng ml<sup>−1</sup>) for the indicated times. Data are representative of two independent experiments. b, UNC93B1–Flag expression levels, as measured by Flag immunoblot, of UNC93B1-deficient RAW macrophages retrovirally transduced to express the indicated UNC93B1 alleles. Cell lines are as described in Fig. 1a. All data are representative of three independent experiments, unless otherwise noted.
Extended Data Fig. 2 | UNC93B1PKP does not alter TLR9 responses, unlike UNC93B1(D34A). a, Representative flow cytometry analysis showing the percentage of TNF-positive cells, measured by intracellular cytokine staining, of indicated RAW macrophage lines after stimulation with CpG-B (25 nM), R848 (10 ng ml\(^{-1}\)), ssRNA (1 µg ml\(^{-1}\)), poly(I:C) (20 µg ml\(^{-1}\)), or LPS (10 ng ml\(^{-1}\)). Shaded histograms show unstimulated controls. b, TNF production, measured by ELISA, from the indicated RAW macrophage lines after stimulation for 8 h with R848 (10 ng ml\(^{-1}\)), CpG-B (25 nM) or LPS (50 ng ml\(^{-1}\)). Data are mean of \(n = 4\) biological replicates pooled from two independent experiments. P values determined by unpaired two-tailed Student’s t test. c, TLR7 and TLR9 trafficking are normal in UNC93B1PKP but not in UNC93B1(D34A) RAW lines. Immunoblot of TLR7-HA and TLR9-HA from lysates of indicated RAW macrophage lines. FL, full-length. All data are representative of three independent experiments.
Extended Data Fig. 3 | UNC93B1 PKP does not alter TLR7 trafficking or localization. **a**, UNC93B1 PKP does not alter TLR7 export rates. Pulse-chase analysis of TLR7 in RAW macrophages expressing wild-type UNC93B1 and UNC93B1 PKP. Cell lysate was immunoprecipitated with haemagglutinin and subjected to a radiolabelled screen and immunoblot. The full-length and cleaved forms of TLR7 are indicated. Asterisk denotes nonspecific band. Data are representative of two independent experiments. **b**, UNC93B1 PKP does not affect TLR7 trafficking to endosomes. Levels of TLR7, LAMP1 and calnexin in whole-cell lysates or lysates of purified phagosomes from the indicated RAW macrophage lines were measured by immunoblot. Representative of three independent experiments. **c**, Colocalization of TLR7–HA (red) and LAMP1 (green) in RAW macrophages expressing the indicated UNC93B1–Flag alleles on a Myd88−/− background using super-resolution structured illumination microscopy. Boxed areas are magnified. The plot shows quantification of the percentage of total TLR7 within LAMP1+ endosomes, with each dot representing an individual cell. Data are mean ± s.d. and pooled from two independent experiments. Scale bars, 10 µm. **d**, The subcellular localization of UNC93B1 PKP is not altered relative to wild-type UNC93B1. Co-localization of UNC93B1–Flag (red) and LAMP1 (green) was measured using super-resolution structured illumination microscopy in UNC93B1-deficient RAW macrophages complemented with wild-type, PKP or H412R mutant UNC93B1. A representative cell is shown for each UNC93B1 allele. Boxed areas are magnified. The plot shows quantification of the percentage of total UNC93B1 within LAMP1+ endosomes, with each dot representing an individual cell. Data are mean ± s.d. and acquired in a single experiment. Scale bars, 10 µm. P values determined by unpaired two-tailed Student’s t-test.
Extended Data Fig. 4 | Mass spectrometry analysis of UNC93B1 complexes. 

a, A small fraction of UNC93B1 resides in endosomes compared to the ER. Subcellular fractionation of RAW macrophages expressing TLR7–HA and UNC93B1–Flag was performed by density-gradient centrifugation. The distribution of calnexin (ER), LAMP1 (late endosomes and lysosomes), UNC93B1–Flag and TLR7–HA across fractions was measured by immunoblot. Data are representative of three independent experiments.

b, Workflow for isolation of phagosomes from RAW macrophages and purification of UNC93B1–Flag complexes from phagosome lysates.
Extended Data Fig. 5 | Syntenin-1 and syntenin-2 inhibit TLR7 signalling.

a, Syntenin-1 is selectively recruited to UNC93B1 after TLR7 stimulation, but not TLR3 stimulation. Syntenin-1 binding to UNC93B1 was measured by Flag immunoprecipitation followed by immunoblot for syntenin-1 from RAW macrophage lines stimulated with R848 (0.5 µg ml\(^{-1}\)) or poly(I:C) (10 µg ml\(^{-1}\)) for the indicated times. Levels of syntenin-1 and UNC93B1–Flag in cell lysates are also shown.

b, Syntenin-1 associates selectively with the TLR7–UNC93B1 complex, but not with TLR9. Syntenin-1 binding to TLR7–HA or TLR9–HA was measured by haemagglutinin immunoprecipitation followed by immunoblot for syntenin-1 from indicated RAW macrophage lines stimulated with R848 (0.5 µg ml\(^{-1}\)) or CpG-B (0.5 µM) for the indicated times. Levels of syntenin-1 and TLR7–HA or TLR9–HA in cell lysates are also shown.

c, NF-κB activation in HEK293T cells transiently expressing syntenin-1 and stimulated with TNF (10 ng ml\(^{-1}\)).

d, NF-κB activation in HEK293T cells transiently expressing TLR7 and increasing amounts of syntenin-2. Cells were stimulated with R848 (50 ng ml\(^{-1}\)) for 16 h before collection. Data in c and d were measured using a dual luciferase reporter assay, normalized to Renilla expression and expressed as RLUs. Data are mean ± s.d., n = 3 biological replicates. *P < 0.05, **P < 0.01 and ***P < 0.001, one-way ANOVA followed by a Tukey’s post-test (95% confidence interval). All data are representative of at least three independent experiments.
Extended Data Fig. 6 | UNC93B1(K333R) confers enhanced TLR7 signalling without affecting TLR9 and TLR3. Flow cytometry analysis showing the percentage of TNF-positive cells, measured by intracellular cytokine staining of UNC93B1-deficient RAW macrophages expressing the indicated alleles after stimulation with CpG (25 nM), R848 (8 ng ml$^{-1}$), poly(I:C) (20 µg ml$^{-1}$), or LPS (10 ng ml$^{-1}$). Shaded histograms show unstimulated controls. Data are representative of three independent experiments.
Extended Data Fig. 7 | Serine phosphorylation in the C-terminal tail of UNC93B1 restricts TLR7 signalling. a, Validation of the anti-phospho-UNC93B1 polyclonal UNC93B1 antibody. a, Immunoblots demonstrating the specificity of the phospho-specific antibodies generated against Ser547 and Ser550 in the UNC93B1 C-tail. Varying quantities of synthesized peptides corresponding to the UNC93B1 C-terminal regulatory region with (P-UNC93B1-C) and without (NP-UNC93B1-C) phosphorylated Ser547 and Ser550 were dropped onto membrane and probed with rabbit phospho-specific, affinity-purified polyclonal anti-UNC93B1 IgG. Data are representative of two independent experiments. b, Phospho-specific polyclonal antibodies detect both phosphorylated Ser547 and Ser550. UNC93B1 was isolated from UNC93B1-deficient RAW macrophages expressing UNC93B1 mutants S547A, S550A or S547A/S550A by Flag immunoprecipitation followed by immunoblot with phospho-specific polyclonal antibodies. Data are representative of at least three independent experiments. c, Intracellular cytokine staining of TNF in macrophage lines expressing the indicated UNC93B1 alleles and stimulated with CpG (10 nM), R848 (10 ng ml⁻¹), ssRNA (1 µg ml⁻¹), poly(I:C) (20 µg ml⁻¹), or LPS (10 ng ml⁻¹). Grey histograms are unstimulated controls. d, TNF production, measured by ELISA, from the indicated RAW macrophage lines after stimulation for 8 h with LPS (50 ng ml⁻¹). Data are mean ± s.d., n = 3 biological replicates. Data are representative of three independent experiments. e, Levels of phosphorylated p38 and JNK, as measured by immunoblot, in lysates of the indicated RAW macrophage cells stimulated with R848 (50 ng ml⁻¹). Data are representative of two independent experiments.
Extended Data Fig. 8 | Genetic variation in the human UNC93B1 C-terminal regulatory region increases TLR7 responses. NF-κB activation in human HEK293T cells transiently expressing TLR7 or TLR5 and the indicated human UNC93B1 alleles was measured using a dual luciferase reporter assay. Cells were stimulated with R848 (10 ng ml⁻¹) or flagellin (2 ng ml⁻¹) for 16 h before collection. Data are normalized to Renilla expression and expressed as RLUs. Data are mean ± s.d., n = 3 biological replicates. *P < 0.05, **P < 0.01 and ***P < 0.001, one-way ANOVA followed by a Tukey's post-test (95% confidence interval). Data are representative of three independent experiments.
Extended Data Fig. 9 | UNC93B1PKP knock-in mice develop systemic inflammation. a, CRISPR–Cas9 strategy to generate UNC93B1PKP knock-in mice. Green line indicates the guide sequence. Red bases indicate the edited codons. A representative sequencing trace of genomic DNA from an edited founder mouse is shown. b, Flow cytometry analysis of the indicated immune cell populations in 6–8-week-old Unc93b1WT/WT, Unc93b1PKP/WT and Unc93b1PKP/PKP mice. Frequencies of dendritic cells (CD11b+CD11c+MHCIIhigh) and inflammatory monocytes (CD11b+Ly6c+Ly6Gneg) in lymph nodes are shown. Data points were pooled from four independent experiments. P values determined by unpaired two-tailed Student’s t-test. 

c, Unc93b1 PKP/PKP mice exhibit signs of emergency granulopoiesis in their bone marrow compartment. Flow cytometry analysis of bone marrow from 6–8-week-old Unc93b1WT/WT, Unc93b1PKP/WT and Unc93b1PKP/PKP mice. Gates representing LSK (CD45+CD3ε−CD19−Ly6c−Ly6G−Sca-1highc-Kithigh) and Sca-1highc-Kit− cells (CD45+CD3ε−CD19−Ly6c−Ly6G−Sca-1highc-Kit−) are indicated and compiled frequencies of Sca-1 highc-Kit− cells are shown on the right. Data are mean ± s.d., n = 3 biological replicates. P values determined by unpaired two-tailed Student’s t-test. d, Representative staining, corresponding to compiled results shown in Fig. 4c, of anti-nuclear antibodies (ANA) using sera from the indicated mouse ages and genotypes. e, f, Flow cytometry analysis showing percentage of TNF-positive cells, measured by intracellular cytokine staining of BMMs and BMDCs derived from the indicated mice after stimulation with CpG-B (150 nM), R848 (10 ng ml−1), ssRNA (1 µg ml−1), poly(I:C) (10 µg ml−1), or LPS (10 ng ml−1). Shaded histograms are unstimulated controls. g, TNF production by BMDCs derived from the indicated mice after stimulation for 8 h with R848, CpG-B (150 nM) or LPS (50 ng ml−1). h, TNF production by BMMs from the indicated mice after stimulation for 8 h with CpG-B (500 nM), LPS (50 ng ml−1), or increasing concentrations of R848. Data in g and h are mean ± s.d., n = 3 biological replicates. P values determined by unpaired two-tailed Student’s t-test. i, B cells from Unc93b1PKP/PKP mice show enhanced proliferation in response to TLR7 stimulation. Proliferation of CFSE-labelled B cells after 3 days stimulation with R848 (8 ng ml−1) or LPS (1.6 µg ml−1) was measured by FACS, pre-gating on live CD19+ cells. The proliferation index is determined by dividing the geometric mean fluorescent intensity (gMFI) of the fluorescent dye CSFE of the unstimulated control by the gMFI of CSFE of the stimulated sample (CSFEunstim/CSFEmulti). Data are mean ± s.d., n = 5 mice per group pooled together from three independent experiments. P values determined by unpaired two-tailed Student’s t-test. j, Immunoprecipitation of MyD88 from BMMs from the indicated mice after stimulation with R848 (500 ng ml−1), followed by immunoblot for IRAK2. Input levels of MyD88 and IRAK2 in whole-cell lysates are also shown. k, UNC93B1 protein levels in BMMs from indicated mouse genotypes, measured by immunoblot with polyclonal antibodies against endogenous UNC93B1. All data are representative of three independent experiments, unless otherwise noted.
Extended Data Fig. 10 | Gating strategies. Representative gating strategies for marginal zone B cells, activated T cells, dendritic cells, inflammatory monocytes, emergency granulopoiesis in bone marrow and B cell proliferation in splenocyte cultures are shown. These strategies were used for the data presented in Fig. 4 and Extended Data Fig. 9.
Reporting Summary

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection
- Immunoblot images: Odyssey Infrared Imaging System (version 2.1), LI-COR Biosciences
- Immunofluorescence staining data: ZEN (version 1.1.2.0), Carl Zeiss
- ELISA data: SoftMax Pro (version 5.0.1), Molecular Devices Corp.
- Flow cytometric data: FACSdiva (version 6.1.3), BD Biosciences
- Luciferase assay data: SoftMax Pro (version 5.4), MDS Analytical Tech.

Data analysis
- Protein identification and quantification by mass spectrometry were done with Integrated Proteomics Pipeline (IPZ), Integrated Proteomics Applications, Inc. San Diego, CA.
- Flow cytometry data was analyzed with FlowJo 10.1.r7.
- Microscopy images were processed in ZEN 2012 1.1.2.0 (Carl Zeiss).
- Colocalization analysis was performed with CellProfiler 2.2.0.
- Immunoblot quantification was performed in Fiji 1.0 (imageJ).
- All other data/figures were plotted and analyzed using Prism 5.0c or 6.0 (GraphPad).
- Statistical analysis was performed with Prism 5.0c, 6.0 or 7.0.

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The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No statistical methods were used to predetermine sample sizes. Sample sizes for studies with mice were determined by availability of animals of the correct genotypes or were based on the numbers used in previous publications, in which comparable sample sizes resulted in statistically significant results. Statistical tests (as described in the Methods) were used to determine statistical significance.

Data exclusions
No data were excluded from analyses.

Replication
Every experiment was repeated (with similar results) at least twice. In most cases, experiments were repeated greater than 3 times with similar results.

Randomization
Randomization was not appropriate for this study. Littermate mice placed into groups based on their genotypes to enable meaningful comparisons.

Blinding
Investigators were blinded to the genotype of samples for analysis of anti-nuclear antibody staining of serum samples.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Unique biological materials |
| ☒   | Antibodies |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq |
| ☒   | Flow cytometry |
| ☒   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Any unique biological resources will be made readily available to any investigators at non-profit institutions after publication and upon request. Requests from for-profit corporations will be handled and negotiated by our university’s technology transfer office. Unique mouse strains will also be made available, given that the investigators provide written assurance that the animals will be used in accordance with their institution’s IACUC guidelines.
**Antibodies**

The following antibodies were used for immunoblot and immunoprecipitations: anti-HA as purified antibody or matrix (clone 3F10; Abcam 118674/3001, 1:100; matrix: 1185016001, Roche), anti-FLAG as purified antibody or matrix (M2; Ab: F1804, 1:2000; matrix: M8823, Sigma-Aldrich), anti-mLamp1 (AF437, 1:1000, R&D Systems), anti-Calnexin (ADI-SPA-860, 1:1000, Enzo Life Sciences), anti-Gadph (GT235, 1:5000, GeneTex), anti-MYD88 (AF3109, 1:ug/ml, R&D Systems), anti-IRAK2 (4367, 1:1000, Cell Signaling), anti-Phospho-p38 (9211, 1:1000, Cell Signaling), anti-p38 (9212, 1:1000, Cell Signaling), anti-Phospho-SAPK/JNK (R381, 1:1000, Cell Signaling), anti-SAPK/JNK (965, 1:1000, Cell Signaling), anti-Phospho-p44/42 (ERK2/1) (D16.14.4E, 1:1000, Cell Signaling), anti-p44/42 (ERK2/1) (1273S, 1:1000, Cell Signaling), anti-IRB (2942, 1:1000, Cell Signaling), anti-Syntelin-1 (J212, 1:25, Novusbio), anti-Unc93B1 (PAS-20510, 1:2000, Thermo Scientific), anti-ubiquitin (P4D1, 1:400, Santa Cruz), anti-k63-linked ubiquitin (human polyclonal, kind gift from Michael Raape, 1:10000), anti-Allx (GTK64975, 1:400, GeneTex), anti-C663 (98217345, 1:2000, Abcam), goat anti-mouse Igg AleeXFluor680 (A21058, 1:10000, Invitrogen), rabbit anti-gogot Igg AleeXFluor680 (A21088, 1:10000, Invitrogen), goat anti-human IRDye 680RD (926-68073, 1:1000, Licor), goat anti-mouse IRDye 800CW (926-32710, 1:1000, Licor), donkey anti-rabbit IRDye 680RD (926-68073, 1:1000, Licor), goat anti-rabbit Igg AleeXFluor647 (711-606-152, 1:500, Jackson Immunoresearch), Cells were mounted in Vectashield Hard Set Mounting Medium for Fluorescence (H-1400, Vector Laboratories).

For ELISA: anti-mouse TNFa purified (F3F3D04, 14-7325-85, 1:ug/ml, ebioscience), anti-mouse TNFalpha-biotin (XT3/XT22, 13-7326-85, 1:ug/ml, ebioscience), Streptavidin-HRP (SA0466, 1:8000, BD Pharmingen).

Antibodies and reagents used for flow cytometry were: anti-CD29, anti-CD9, anti-CD18, anti-CD29, 17-3232-82, 1:500, ebioscience), purified anti-CD16/32 Fr Block (2.452, 553341, 1:500, BD), CD3 (145-2C11, 100327, 1:100, Biolegend), CD4 (GK1.5, 10453, 1:800, Biolegend), CD8a (53-6-7, 100742, 1:800, BioLegend), CD44 (478, 1:041-85, 1:100, ebioscience), CD62L (MEL-14, 12-0623-81, 1:400, ebioscience), CD69 (H1.2F3, 25-0691-82, 1:400, ebioscience), CD16 (53-7.3, 17-0011-82, 1:200, ebioscience), B220 (RA3-682, 56-0452-82, 1:200, Invitrogen), CD19 (1D5, 115555, 1:800, Biolegend), IgD (12-26c2a, 40704, 1:200, Biolegend), IgM (eB121-15F9, 25-8590-82, 1:200, ebioscience), CD21 (eB9, 47-0211-82, 1:200, ebioscience), CD23 (33B4, 101618, 1:200, ebioscience), CD152 (1H10.1, 12450, 1:200, Biolegend), CD11b (M1/70, 101245, 1:1000, Biolegend), CD4 (24G2, 12402, 1:1000, Biolegend), CD8 (53.6.7, 14502, 1:1000, Biolegend), CD11c (48-7A12, 12402, 1:1000, Biolegend), CD11c (48-7A12, 12402, 1:1000, Biolegend), CD11c (48-7A12, 12402, 1:1000, Biolegend).

The antibody against phosphorylated Unc93B1 was generated by Invitrogen against synthesized phospho-peptide (VELEODnpS(Sp5)EGEGLQ using the “Rabbit, 90 Day Immunization” protocol. Antibody in sera was enriched with immobilized phosphopeptide, followed by negative absorption with unphosphorylated peptide.

**Validation**

The custom-designed rabbit polyclonal antibody against phospho-Unc93B1 for IHC studies was validated in Extended Data Fig. 7a and b. The custom-designed human polyclonal antibody against k63 ubiquitin for IHC studies was validated previously and published in https://doi.org/10.1016/j.cell.2017.09.040. All other commercial antibodies were validated by manufacturers for indicated species and application. Antibodies used for flow cytometry and immunofluorescence microscopy were further validated by staining known populations of cells. When possible, genetic controls were used to confirm specificity of any staining. Antibodies used for biochemistry were further validated by performing pilot experiments using samples with known parameters.

**Eukaryotic cell lines**

| Policy information about: cell lines |
|-------------------------------------|
| Cell line source(s): RAW264 macrophage cell line was obtained from ATCC. HKE293 cells were obtained from ATCC. |

**Authentication**

Cells lines were authenticated via morphology, flow cytometry (measuring surface markers), and functional assays. Individual lines were not kept in culture for longer than 2 months.

**Mycoplasma contamination**

Cells lines were screened for mycoplasma. Also, individual lines were not kept in culture for longer than 2 months. New lines were thawed from cryopreserved stocks previously confirmed as mycoplasma-negative.

**Commonly misidentified lines**

None of the cell lines used are listed in the ICILC database.

**Animals and other organisms**

| Policy information about: studies involving animals, ARRIVE guidelines recommended for reporting animal research |
|---------------------------------------------------------------|
| Laboratory animals: Mouse, C57BL/6, males and females, ages ranging from 3 weeks to greater than 5 months. Mouse, Unc93b1(KP) knockin mouse, males and females, ages ranging from 3 weeks to greater than 5 months. Mouse, Ilr7-/- (on the C57BL/6 background), males and females, ages ranging from 3 weeks to greater than 5 months. |

Wild animals: The study did not involve wild animals.
Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Toll-like receptor signaling in RAW macrophages, BMMs, and BM-DCs: Cells were seeded into non-treated tissue culture 24-well plates or round-bottom 96-well plates. The next day cells were stimulated with the indicated TLR ligands. To measure TNFα production, Brefeldin A (BD GolgiPlug, BD Biosciences) was added to cells 30 min after stimulation, and cells were collected after an additional 5.5 h. Dead cells were excluded using a fixable live/dead stain (Violet fluorescent reactive dye, Invitrogen). Cells were stained for intracellular TNFα with a Fixation & Permeabilization kit according to manufacturer’s instructions (eBioscience). (BM-DCs were additionally surface-stained for CD11c, MHCII, and Ly6c). Mouse characterization: Spleens and lymph nodes were digested with collagenase XI and DNase I for 30 min at 37°C and single cell suspensions were generated by mechanical disruption. Red blood cells were lysed in ACK Lysing Buffer (Gibco). The remaining cells were stained for flow analysis. Dead cells were excluded using a fixable live/dead stain (Aquarius fluorescent reactive dye, Invitrogen) or DAPI and all stains were carried out in PBS containing 1% BSA (w/v) and 0.1% Azide (w/v) including anti-CD16/32 blocking antibody. Cells were stained for 20 min at 4°C with surface antibodies. Data were acquired on a Fortessa or X20 (BD Biosciences).

For proliferation assays, spleens were digested with collagenase B (Sigma) and DNase I for 45 min and red blood cells were lysed using ACK buffer (Gibco). Splenocytes were labeled with 12.5ug/ml CFSE (Invitrogen) for 10min at 37°C and immediately underlaid with 3ml FCS to spin out CFSE. Cells were taken up in media (RPMI/10%FCS/L-glutamine/Pen-Strep/HEPES/Sodium pyruvate/ME), counted, and seeded at 200,000 cells per well in round-bottom 96-well plates. Cells were incubated in media with various concentrations of CpG-B, R848, or LPS for 72 h. Flow cytometry was used to analyze stimulated cells. Live, singlet cells were pre-gated on CD19+ and cell proliferation was determined by the geometric mean fluorescence intensity (gMFI) of CFSE. For the quantification, the gMFI CSFE of the unstimulated control was divided by the gMFI CSFE of the stimulated sample (CSFEUnstim:CSFESample) and plotted along the ligand titration.

Instrument
BD Fortessa or X20 flow cytometer (BD Biosciences)

Software
FlowJo 10.1r7

Cell population abundance
Unc93b1 / Raw macrophages retrovirally transduced with Unc93b1 mutants were sorted on mCherry to 95-99% purity.

Gating strategy
All cells, including RAW macrophages, BMMs, BM-DCs, B cells, bone marrow cells, and mouse splenocytes and lymph node cells were gated in the following order to determine the frequencies of live cells:1) FSC-A/SSC-A, 2) LIVE/dump, 3) Singlets (FSC-H/FSC-A). Data concerning the frequency of different cell subsets were reported as frequency of live cells (unless otherwise stated). The precise gating strategy for each immune cell type is outlined in Fig. S8. For Intracellular TNFα staining, the frequencies of TNFα positive cells of live cells are displayed.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.