Figures and figure supplements

FRMD8 promotes inflammatory and growth factor signalling by stabilising the iRhom/ADAM17 sheddase complex

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Figure 1. FRMD8 is a novel interaction partner of iRhom1 and iRhom2. (A) Volcano plot representing results from three iRhom2 co-immunoprecipitations. The fold change of label-free quantification values (in log2 ratio) was plotted against the p value (-log10 transformed). The grey dotted line indicates p-values <0.05 (analysed with a two-sample t-test). Benjamini-Hochberg correction was applied to adjust the p-value for multiple hypothesis testing (dark grey dotted line). (B) Lysates of HEK293T cells stably expressing human iRhom1-3xHA or iRhom2-3xHA transfected with human FRMD8-V5 (where indicated) were subjected to anti-HA and anti-V5 immunoprecipitation (HA-IP, V5-IP) and a western blot using anti-HA and anti-V5 antibodies was performed. Black arrowheads indicated the co-immunoprecipitated FRMD8-V5; white arrowheads indicated the co-immunoprecipitated iRhoms.

DOI: https://doi.org/10.7554/eLife.35012
Figure 1—figure supplement 1. Setup and confirmation of the mass spectrometry screen. (A) HEK293T cells transiently transfected with human iRhom2-3xHA or UNC93B1-3xHA were stained with DAPI (blue) to label nuclei, anti-HA to label iRhom2-HA (red), and anti-calnexin to label the ER (green). Scale bar = 10 μm. (B) Lysates and anti-HA immunoprecipitation (HA-IP) from wild-type (WT) and FRMD8 knockout (KO) HEK293T cells stably expressing iRhom2-3xHA (where indicated) were immunoblotted for HA and FRMD8. Nonspecific bands are marked with an asterisk.

DOI: https://doi.org/10.7554/eLife.35012.004
Figure 2. FRMD8 loss reduces mature ADAM17 levels and impairs ADAM17-dependent shedding activity. (A) ADAM17 levels were analysed in HEK293T cells transfected with non-targeting siRNA control pool (ctrl) or FRMD8 SMARTpool siRNA after western blotting with anti-ADAM17 and anti-actin staining. In this and subsequent figures, pro- and mature form of ADAM17 are indicated with black and white arrowheads, respectively. Lower panel: Knockdown efficiency of FRMD8 was analysed by TaqMan PCR. (B, C) Lysates from wild-type (WT) and FRMD8 knockout (KO) HEK293T cells, transiently transfected with FRMD8-V5 for 72 hr (where indicated) and immunoblotted for endogenous ADAM17, ADAM10, FRMD8 and actin using western blotting. Nonspecific bands are marked with an asterisk. (D) Cell surface levels of endogenous ADAM10 and ADAM17 were analysed in WT and FRMD8 KO HEK293T cells after stimulation with 200 nM PMA for 5 min. Unpermeabilised cells were stained on ice with ADAM10 and ADAM17 antibodies, or only with the secondary antibody as a control (grey). The immunostaining was analysed by flow cytometry. The graph shown is one representative experiment out of four biological replicates. The geometric mean fluorescence was calculated for each experiment using FlowJo software. Statistical analysis was performed using an unpaired t-test. (E, F) WT and FRMD8 KO HEK293T cells were transiently transfected with alkaline phosphatase (AP)-tagged AREG, HB-EGF or TGFα, and then either incubated with 200 nM PMA, with 200 nM PMA and 1 μM GW (ADAM10/ADAM17 inhibitor), or with DMSO for 30 min. In addition, cells transfected with AP-TGFα were either left unstimulated for 20 hr or incubated with GW for 20 hr. AP activity was measured in supernatants and cell lysates. Each experiment was performed in biological triplicates. The results of three independent shedding experiments are shown. Statistical analysis was performed of using a Mann-Whitney test. ns = p value>0.05; *=p value<0.05; ***=p value<0.001; ****=p value<0.0001.

DOI: https://doi.org/10.7554/eLife.35012.006
Figure 3. FRMD8 binds to the iRhom2 N-terminus. (A) Schematic representation of truncated human iRhom2 constructs used in (B–E). (B, C) Lysates and anti-HA immunoprecipitation (HA-IP) from HEK293T cells transiently co-transfected with FRMD8-V5 and either empty vector (vect) or truncated human iRhom2-3xHA constructs were immunoblotted for V5 and HA. (D) iRhom1/2 double knockout HEK293T cells stably expressing empty vector (vect) or human iRhom2-3xHA constructs were transiently transfected with alkaline phosphatase (AP)-tagged AREG and then incubated with 200 nM PMA or with DMSO for 30 min. AP activity was measured in supernatants and cell lysates. Each experiment was performed in biological triplicates. The results of three independent shedding experiments are shown. Statistical analysis was performed using a Mann-Whitney test. ****=p value<0.0001. (E) Lysates from iRhom1/2 double knockout HEK293T cells transiently transfected with empty vector (vect) or human iRhom2-3xHA constructs were immunoblotted for ADAM17 and HA.

DOI: https://doi.org/10.7554/eLife.35012.007
Figure 3—figure supplement 1. A) Amino acid sequence alignment of human and mouse iRhom2 N-terminal region using Clustal Omega. The region required for FRMD8 binding is highlighted in red. Conserved phosphorylation sites that have been mutated to alanine in the iRhom2pDEAD (Figure 10—figure supplement 1) are marked in yellow. Grey residues indicate additional phosphorylation sites that have been reported on PhosphoSitePlus (www.phosphosite.org). An asterisk (*) indicates positions which have a fully conserved residue, a colon (:) indicates strongly similar properties of the amino acids, and a period (.) indicates weakly similar properties according to the Clustal Omega tool. (B) Lysates and anti-HA immunoprecipitation (HA-IP) from HEK293T cells transiently transfected with FRMD8-V5 and either empty vector (vect), mouse iRhom2WT (WT) or Rhom2cub (D268) were immunoblotted for V5 and HA.

DOI: https://doi.org/10.7554/eLife.35012.008
Figure 4. iRhom2 binds to FRMD8 and ADAM17 simultaneously. (A) Lysates, anti-HA and anti-V5 immunoprecipitations (HA-IP, V5–IP) of HEK293T cells co-expressing human iRhom2-3xHA and human FRMD8-V5 were immunoblotted for ADAM17, HA and V5. (B) Lysates of wild-type (WT) and ADAM17 knockout (KO) HEK293T cells were transiently transfected with human iRhom2-3xHA and FRMD8-V5 (where indicated), anti-HA and anti-V5 immunoprecipitated (HA-IP; V5–IP) and immunoblotted for ADAM17, HA, and V5. (C) Lysates of WT and FRMD8 KO HEK293T cells stably expressing human iRhom2-3xHA were anti-HA immunoprecipitated (HA-IP) and stained for ADAM17 and HA. Nonspecific bands are indicated by an asterisk. (D) Lysates of WT and iRhom1/2 double knockout (DKO) HEK293T cells stably expressing human iRhom2WT-3xHA or iRhom2D201-300-3xHA were anti-V5 immunoprecipitated (V5–IP) and immunoblotted for ADAM17, HA and V5.

DOI: https://doi.org/10.7554/eLife.35012.009
Figure 5. FRMD8 promotes cell surface localisation of iRhom2. (A, B) Immunofluorescence of iRhom1/2 double knockout HEK293T cells stably expressing iRhom2-3xHA or iRhom2\textsuperscript{D300}-3xHA and transiently transfected with FRMD8-V5.
Figure 5 continued

FRMD8-V5 for 72 hr. Cells were stained for HA (red), V5 (green) and DAPI for DNA (blue). Single confocal sections are shown, taken through the centre of the nucleus. (C) Schematic model of the FRMD8-iRhom2D300 construct used in (E). (D, E) Immunofluorescence of iRhom1/2 double knockout HEK293T cells stably expressing iRhom2D300-3xHA or FRMD8-iRhom2D300-3xHA and transiently transfected with ADAM17-V5 for 72 hr. Cells were stained for HA (green), V5 (red) and DAPI for DNA (blue). Single confocal sections are shown, taken either through the centre of the nucleus (MEDIAL), or at basal regions close to the coverslip (BASAL). In all images the scale bar = 10 μm.

DOI: https://doi.org/10.7554/eLife.35012.010
Figure 6. FRMD8 loss leads to degradation of iRhom2 and mature ADAM17 through the lysosomal pathway. (A–D) Immunofluorescence of iRhom1/2 double knockout HEK293T cells stably expressing iRhom2-3xHA or iRhom2Δ300-3xHA treated with DMSO (CON) or 100 nM bafilomycin A1 (BAF) for 16 hours. Figure 6 continued on next page.
Figure 6 continued

hr prior to fixation. Cells were stained for HA (green), the lysosomal marker LAMP1 (red) and DAPI for DNA (blue). LAMP1-labelled regions (within white boxes) have been magnified. Scale bar = 10 μm. (E, F) iRhom2	extsuperscript{D300-3xHA} cells were treated as in (A–D), but with 72 hr expression of ADAM17-V5 and labelling of HA (green), V5 (red) and DAPI for DNA (blue). Arrows indicate colocalising puncta. Single confocal sections are shown, taken through the centre of the nucleus. HA- and V5-labelled regions (within white boxes) have been magnified. Scale bar = 10 μm. (G) Cell lysates of wild-type (WT) and FRMD8 knockout (KO) HEK293T cells treated with the solvent DMSO (−), 10 μM MG-132 (MG) or 200 nM bafilomycin A1 (Baf) for 16 hr were enriched for glycosylated proteins using concanavalin A (conA) beads and immunoblotted for ADAM17 and transferrin receptor 1 (TIR). TIR was used as a loading control although it is also susceptible to bafilomycin treatment. Mature ADAM17 levels from three experiments were quantified relative to TIR levels using ImageJ.

DOI: https://doi.org/10.7554/eLife.35012.011
Figure 7. FRMD8 loss leads to the destabilisation of ADAM17 and iRhom2. (A) Unpermeabilised WT (black) and FRMD8 KO HEK293T (cyan) cells stably expressing human iRhom2-3xHA were immunostained on ice for HA. Wild-type HEK293T cells immunostained for HA served as a negative control (grey). (B) Cells were permeabilised and stained at room temperature with an anti-HA antibody. Immunostaining with the Alexa Fluor 488-coupled secondary antibody served as a control (grey). The flow cytometry graphs shown are one representative experiment out of three experiments. The geometric mean fluorescence was calculated for each experiment using FlowJo software. Statistical analysis was performed using an unpaired t-test; ns = p value>0.05; *=p value<0.05. (C) Lysates of HEK293T cells stably expressing human iRhom2-3xHA and transiently transfected with FRMD8-V5
Figure 7 continued

(Where indicated) were analysed by western blot for iRhom2 levels using anti-HA, anti-V5 and anti-actin immunostaining. Nonspecific bands are marked with an asterisk. (D) Lysates of WT and FRMD8 KO HEK293T cells stably expressing human iRhom2-3×HA (where indicated) were immunoblotted for HA, FRMD8 and actin. An asterisk marks nonspecific bands. (E) FRMD8 mRNA levels relative to actin mRNA levels were determined by TaqMan PCR in cells used in (D).

DOI: https://doi.org/10.7554/eLife.35012.012
Figure 7—figure supplement 1. FRMD8 stabilises iRhom levels by preventing its lysosomal degradation. (A) iRhom1/2 double knockout HEK293T cells stably expressing iRhom2<sup>WT</sup>-3xHA, iRhom2<sup>Δ300</sup>-3xHA or FRMD8-iRhom2<sup>Δ300</sup>-3xHA were treated with 100 μg/ml cycloheximide (CHX) for the indicated time (0–8 hr) to block protein synthesis. Cell lysates were immunoblotted for HA and actin. (B) Cell lysates of wild-type (WT) and FRMD8 knockout (KO) HEK293T cells treated with 10 μM MG-132 (MG), 200 nM bafilomycin A1 (Baf) or 50 mM ammonium chloride (NH<sub>4</sub>Cl) for 16 hr were immunoblotted for ADAM17, FRMD8, and actin. An asterisk marks a nonspecific band. (C) N-glycosylation of iRhom2 was analysed using EndoH and PNGase to distinguish ER/cis-Golgi (EndoH sensitive) and late Golgi localisation (EndoH resistant). Lysates of WT and FRMD8 KO HEK293T cells transiently transfected with mouse iRhom2-3xHA were deglycosylated with EndoH or PNGase and then immunoblotted for mouse iRhom2, human FRMD8 and actin. An asterisk marks a nonspecific band. (D) Lysates of HEK293T cells stably expressing human iRhom1-3xHA and transfected with FRMD8-V5 (where indicated) were immunoblotted for HA, V5, and actin. (E) Levels of ADAM17 were analysed in HEK293T-iRhom2-3xHA and HEK293T WT cells transfected with siRNAs targeting iRhom2 where indicated. Cell lysates were immunoblotted using an anti-ADAM17 or anti-actin antibody. An asterisk marks a nonspecific band.

DOI: https://doi.org/10.7554/eLife.35012.013
Figure 8. FRMD8 stabilises endogenous iRhom2. (A, B) Levels of endogenously 3xHA tagged iRhom2 were analysed in HEK293T-iRhom2-3xHA cells transfected with FRMD8-V5 plasmid, siRNAs targeting iRhom2, non-targeting siRNA control pool (ctrl) or FRMD8 SMARTpool siRNA. Cell lysates were anti-HA immunoprecipitated (HA-IP) to detect endogenous iRhom2-3xHA levels and immunoblotted using anti-HA antibody. Cell lysates were immunoblotted for ADAM17, V5, and actin. (C) FRMD8 and iRhom2 mRNA levels relative to actin mRNA levels were determined by TaqMan PCR in cells used for the experiment shown in (B) to demonstrate that the destabilisation of endogenous iRhom2 was not induced by a change in iRhom2 mRNA levels. 
DOI: https://doi.org/10.7554/eLife.35012.014
Figure 9. FRMD8 is required for iRhom2/TACE regulation in human iPSC-derived macrophages and mice. (A) Schematic representation of the differentiation protocol of iPSCs into macrophages based on (van Wilgenburg et al., 2013). Scale bars = 10 μm. (B) Lysates of iPSC-derived macrophages (on day seven after harvest from EBs) were immunoblotted for ADAM17, FRMD8, and actin. Western blots from three experiments were quantified using ImageJ with actin serving as the loading control. (C) 25,000 iPSC-derived macrophages were either left unstimulated or stimulated with 50 ng/ml LPS for 4 hr. TNFα concentration in the cell supernatants was measured by ELISA and then normalised to the protein concentration in macrophage cell lysates to adjust the cytokine release for potential differences in cell numbers. Each experiment was performed in biological triplicates. Data from three independent experiments were statistically analysed using a Mann-Whitney test; ***=p value<0.001; ****=p value<0.0001. (D, E) Lysates from tissues derived from Frmd8\(^{-/-}\) or Rhbd2\(^{-/-}\) and their wild-type littermates were immunoblotted for ADAM17, FRMD8, iRhom2 and actin. Blots from three experiments using three different littermates of Frmd8\(^{-/-}\) and Frmd8\(^{+/+}\) mice were quantified using ImageJ with actin serving as the loading control. DOI: https://doi.org/10.7554/eLife.35012.015
Figure 9—figure supplement 1. Generation of FRMD8 knockout iPSCs and iPSC-derived macrophages. (A) Sequencing of the genomic DNA isolated from clonal FRMD8 KO iPSCs shows a 1-nt insertion (clone 1) and a 7-nt and 10-nt deletion (clone 2). The targeting sequence of the sgRNA is shown in Figure 9—figure supplement 1 continued on next page.
bold; small letters indicate the sequence within the intronic region; the protospacer adjacent motif (PAM) sequence underlined. (B) Parental wild-type and FRMD8 KO iPSC lines were karyotyped by SNP array. Detected copy number variations are indicated in red (DNA copy number loss in the indicated region) and green (DNA copy number increase). The AH017-13 iPSC line used was derived from a female donor, therefore the Y chromosome is marked in red (loss of Y chromosome DNA). (C) 25,000 iPSC-derived macrophages were either left unstimulated, stimulated with 50 ng/ml LPS, or with 50 ng/ml LPS and simultaneously with 2 μM Gl or 2 μM GW for 4 hr. TNFα concentration in the cell supernatants was measured by ELISA and then normalised to the protein concentration in macrophage cell lysates to adjust the cytokine release for potential differences in cell numbers. Each experiment was performed in biological triplicates. Data from three independent experiments were statistically analysed using a Mann-Whitney test; ns = p value>0.05; ****=p value<0.0001. (D) TNFα mRNA levels relative to actin mRNA levels were measured by TaqMan PCR in WT and FRMD8 KO iPSC-derived macrophages without stimulation and after stimulation with 200 ng/ml LPS for 0.5 hr.

DOI: https://doi.org/10.7554/eLife.35012.016
Figure 9—figure supplement 2. Generation of Frmd8 knockout mice. (A) Schematic representation of the insertion of a lacZ/neomycin cassette into the Frmd8 locus in the ES cells used to generate Frmd8−/− mice. (B) Offspring of Frmd8+/− × Frmd8+/− (HET x HET) crosses listed by genotype: Frmd8+/− (WT), Frmd8+/− (HET), and Frmd8−/− (KO). Two Frmd8 mouse strains were bred (both in BL6 background): one with the entire lacZ/neomycin cassette inserted and one strain in which the neomycin resistance gene has been removed from the cassette. (C) Lysates from skin derived from Frmd8−/−; RhbdZ−/− mice and their wild-type littermate were immunoblotted for iRhom2 and actin.

DOI: https://doi.org/10.7554/eLife.35012.017
Figure 10. FRMD8 stabilises the iRhom2/ADAM17 sheddase complex at the cell surface. Schematic representation of the role of FRMD8 in the iRhom2/ADAM17 pathway: under wild-type conditions ADAM17 and iRhom2 are stabilised by FRMD8 and thereby protected from degradation through the endolysosomal pathway. DOI: https://doi.org/10.7554/eLife.35012.018
Figure 10—figure supplement 1. Lysates and anti-HA immunoprecipitation (HA-IP) from HEK293T cells transiently transfected with human FRMD8-V5 and mouse iRhom2<sup>WT</sup> (WT) or iRhom2<sup>pDEAD</sup> (pDEAD) were immunoblotted for V5 and HA. Where indicated cells have been stimulated with 200 nM PMA for 30 min.
DOI: https://doi.org/10.7554/eLife.35012.019