Mammalian iRhoms have distinct physiological functions including an essential role in TACE regulation

Yonka Christova, Colin Adrain, Paul Bambrough, Ashraf Ibrahim, and Matthew Freeman

Supplemental Methods

Reagents

The following reagents were used: 1,10-phenanthroline and PMA (Sigma), bombesin (Cambridge Bioscience); TACE fluorogenic peptide (ANASPEC, 72085);

Antibodies

The following rabbit polyclonal antibodies were used: TACE (ABCAM Ab39162), ADAM9 (D64B5, Cell Signaling), ADAM10 (ABCAM Ab1997). TACE chicken polyclonal used in immunoprecipitations was a gift of Gillian Murphy, CRUK; chicken IgY, pan-cadherin and tubulin antibodies were from Sigma; Transferrin Receptor (Invitrogen, 13-6800); EGFR (Millipore, clone 20G3); HA-HRPO (Roche), V5-HRPO (Invitrogen), Myc-HRPO (Abcam), FLAG-HRPO (Sigma), GFP-HRPO (Myltenil). Anti-HA affinity resin was from Sigma.

Generation of mouse knockout in iRhom1

As a source of genomic DNA we used PAC clone 588-N14 identified after screening of RP21 mouse genomic library (Source BioScience). LoxP sites flanking exons 2-18 were introduced by recombineering (Liu *et al.*, 2003) and the whole coding region of iRhom1 was deleted by expressing cre recombinase in bacteria. The linearised (NotI) targeting construct was electroporated into EK.CCE embryonic stem (ES) cells derived from 129/SvEv mice. The construct contained a PGK-driven Neo cassette and a MC1 promoter-driven HSV-TK cassette, allowing for positive and negative selection. The ES cells were maintained over a feeder layer of MEFs in the presence of leukaemia inhibitory factor, maintaining the ES cells undifferentiated. After selection with G418 (400 µg /ml) for 8-11 days and Gancyclovir (2.5 µM) for 5 days the surviving clones were screened by Southern blotting using a PCR generated 3' flanking probe in order to confirm the site-specific integration of the loxP sites (Fig S1 A, B). The targeted ES cells from two independent clones were injected into

blastocysts derived from C57BL6 mice and implanted into pseudo-pregnant female ICR mice using standard techniques. Resulting heterozygous iRhom1 +/- chimeric animals were crossed to 129S6/SvEvTac mice to obtain mice on a pure 129S6/SvEvTac background, or to C57BL/6J mice to create the founders of the mixed genetic background. Subsequently, some mice were backcrossed for 10 generations to C57BL/6J. The loss of the iRhom1 genomic locus and loss of expression in animals was confirmed by PCR and RT-PCR (data not shown). Mice used in experiments were sex and age matched and kept in individually ventilated cages in pathogen free conditions. All animal experiments outlined in this study were performed under an appropriate UK Home Office project licence. As iRhom1 knockout animals die before or shortly after weaning, animal weight was monitored. Mice were sacrificed when 20% of the maximum body weight was lost.

Shedding assays

A chimeric version of HB-EGF was generated by fusing *Gaussia* luciferase to a truncation of human HB-EGF lacking the first 84 amino acids. MEFs were transfected with 1 µg of HB-EGF-luciferase or unmodified luciferase, as indicated above. One day later, cells were washed twice in PBS and incubated in 800 µl Optimem (Invitrogen) for 1 hour. Cells were then stimulated by the addition of PMA (250 nM) for 1 hour. Luciferase activity in the supernatants or in lysates generated using TX-100 lysis buffer was determined using the Biolux Gaussia Luciferase assay kit (New England Biolabs). The percentage of the total material shed from each well (i.e, signal from supernatant/lysate+supernatant) was then used to calculate the percentage stimulated induction as indicated:

(%Stimulated-%unstimulated/%unstimulated) x100

This calculation subtracts the signal due to the constitutive shedding of HB-EGF, which does not depend on TACE [Sahin et al., 2004]. To assess GPCR-triggered shedding, cells were additionally transfected with a plasmid encoding mouse Gastrin Releasing Peptide Receptor (GRPR). Cells were stimulated with 1 µM bombesin as described for PMA stimulation.

To assess the secretion of unmodified luciferase, the percentage of total material secreted from unstimulated cells was calculated as indicated above.

Supplemental Figure Legends

Figure S1 Generation of iRhom1 knockout mice

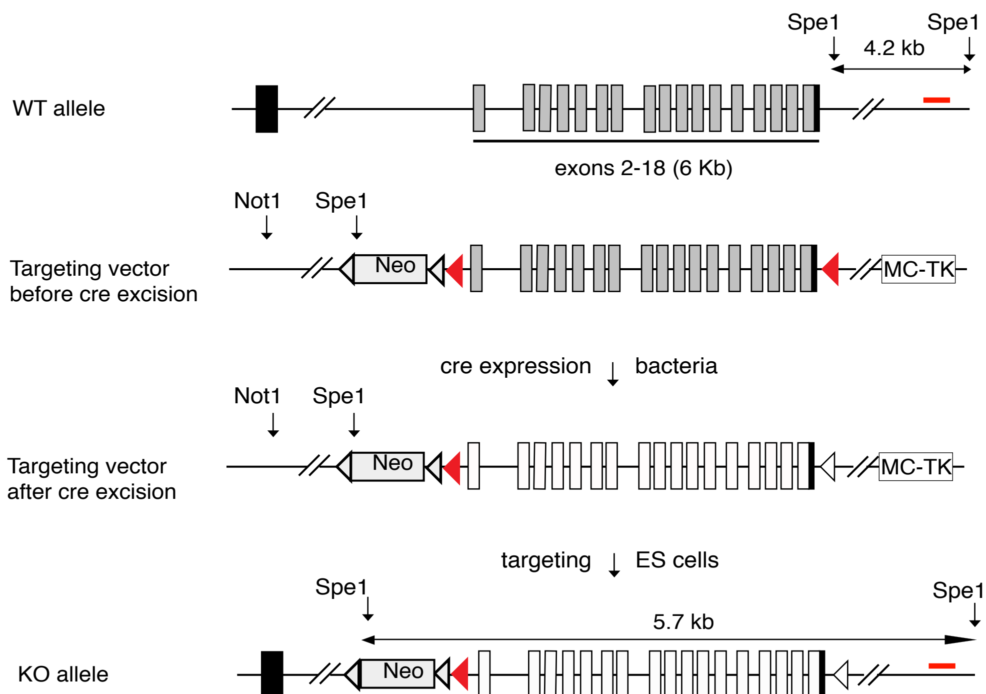
(A) Overview of the targeting strategy. Map of the mouse iRhom1 (also called rhbdf1) genomic locus comprising exons 1-18 (top). Grey bars represent coding exons, black bars represent non coding exons or UTR. Initially, the targeting vector contained loxP sites (red arrow heads) flanking exons 2-18 and a PGK Neo cassette flanked by FRT sites (white arrow heads). The exons deleted by expression of Cre recombinase in bacteria prior to targeting of the ES cells are indicated by white bars. The lonely loxP site replaced the Spe1 site in the WT locus and in addition to this the targeted iRhom2 locus harbours an extra Spe1 site resulting in a 5.7 kb Spe1 fragment as opposed to the 4.2 kb fragment in the WT allele, when detected with a 3' flanking probe (indicated by a red line). (B) Southern blot of Spe1-digested genomic DNA from WT and heterozygous (+/-) ES cells showing an additional 5.7 kb fragment) in the +/- ES cells demonstrating site specific integration of the targeting construct.

Figure S2 ADAM7 trafficking unaffected in double knockout MEFs

Deglycosylation of ADAM7-V5-expressing lysates from WT versus DKO MEFs. The upper, endoglycosidase H-resistant band, indicated by a black arrow, demonstrates that ADAM7 transit from the ER is unaffected in iRhom DKO MEFs.

Figure S1

A



B

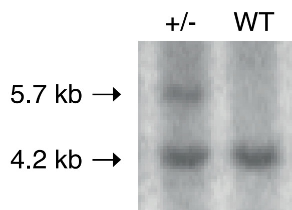


Figure S2