**TNF signalling requires iRhom2 to promote trafficking and activation of TACE**

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**Supplemental Online Material**

## 1. Materials and Methods

### Reagents

The following reagents were used: 1,10-phenanthroline (Sigma); LPS E.coli O111:B4 (InvivoGen); TACE fluorogenic peptide (ANASPEC, 72085); batimastat (BB94); furin inhibitor, decanoyl-RVKR-CMK (EMD biosciences).

### Antibodies

The following antibodies were used: TACE (Ab39162) and ADAM10 (Ab1997) rabbit polyclonal antibodies (ABCAM); a TACE chicken polyclonal (gift of Gillian Murphy, CRUK); chicken IgY (Sigma), pan-cadherin and tubulin antibodies were from Sigma; TNF (R&D, AF-410-NA); p97 (Thermo, MA1-21412); Transferrin Receptor (Invitrogen, 13-6800); EGFR (Millipore, clone 20G3); PERK (Cell Signaling Technology clone C33E10); anti-strep tag (Qiagen); V5-HRPO (Invitrogen). Anti-HA affinity resin and HA peptide were from Sigma, HA-HRPO was from Roche. TNF and IL6 ELISA kits and the Fluorokine Multi Analyte Cytokine Analysis Kits were from BD Bioscience. An easylink HRP conjugation kit (Abcam 39162) was used to conjugate HRP to anti-TACE and anti-iRhom2 polyclonal antibodies (see below).

Anti-iRhom2 polyclonal antibodies were generated by immunizing rabbits with purified, bacterially expressed N-terminal His/GST-tagged fusion protein comprising

mouse iRhom2 N-terminus (amino acids 1-373). The antiserum was depleted of anti-GST antibodies using nitrocellulose-immobilized GST protein, after which it was affinity purified against nitrocellulose membrane-immobilized GST-iRhom2, acidic glycine eluted and neutralized.

**Generation of iRhom2 knock-out mice**

The knockout targeting strategy is outlined in Fig S1A. As a source of genomic DNA we used PAC clone 434-J11 identified after screening of RP21 mouse genomic library (Geneservice). LoxP sites flanking exons 4-9 were introduced by recombineering (*23*) and the linearised (HpaI) targeting construct was electroporated into EK.CCB embryonic stem (ES) cells derived from 129/SvEv mice. The construct contained PGK-driven Neo cassette and 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). Two positive clones were selected and the genomic DNA between the two loxP sites in each clone was excised by expression of cre recombinase in ES cells. The excision of exons 4-9 was confirmed by the loss of G418 resistance and by PCR (for strategy and results see Fig S1) (using primers x, GGTAGAAAGGTGCTGAGTGGCAAGTG; y, GCAGAGTTGAGGACATAGCCCAGACC and z, GAACTCATAGACCTCTGTCTGCCTCTGC). 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 iRhom2 +/- chimeric animals were backcrossed for 8 generations to C57BL/6. The loss of the iRhom2 genomic locus and loss of expression in animals was confirmed by PCR and RT-PCR (Fig S1 C and D). 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 undertaken with the approval of the UK Home Office.

**Macrophage (BMDM) Cell culture**

Bone marrow was flushed from femurs into serum free RPMI (Gibco), centrifuged at 1500 rpm for 10 minutes, and resuspended in macrophage growth media (RPMI 1640 with Glutamax, containing penicillin, streptomycin, gentamycin (10 μg/ml), 50 μM 2-mercaptoethanol 10% FCS, and 20% of a conditioned culture supernatant from confluent L929 cells). Cells were maintained in this medium and were differentiated over 7-14 days. This resulted in a 95% pure macrophage population as determined by flow cytometry with a CD11b antibody (eBioscience). When confluent, or prior to plating for an experiment, cells were detached using a cell scraper after incubating on ice in cell dissociation solution (Sigma).

**Quantitative RT PCR measurements of mRNA levels**

Following RNA extraction from LPS-treated macrophages using the RNeasy kit (Qiagen), cDNA was synthesized using the Superscript III kit (Invitrogen) based on 400ng of total RNA per 10 μl reaction. qPCR was performed on the resultant cDNA using Gene Expression Mastermix (Applied Biosystems), with probes for mouse b-Actin (Mm00607939_s1), iRhom1/RHBDF1 (Mm00711711_m1), iRhom2/RHBDF2 (Mm00553469_m1) and TNF (Mm00443258_m1). The levels of *iRhom1* or *iRhom2* were normalized relative to the beta-actin mRNA levels in each sample; subsequently, *iRhom1* or *iRhom2* mRNA levels in the various treatments were expressed as a

fraction of the 0h untreated sample. For testing induction of TNF mRNA upon LPS exposure, mRNA levels were normalized relative to TBP mRNA (Mm01277045_m1).

***In vivo* LPS induction of cytokines**

For serum measurements of cytokines a tail pre-bleed was taken prior to i.v. injection of 140 μg LPS per mouse. Blood was collected after 3 and 6 hours and serum was subjected to Multi Analyte cytokine profiling for IL-1β, IL-6, IL-12 (p70) and TNF according to the manufacturer's instructions (R&D Systems, Fluorokine MAP) and analysed on a Bio-Plex Protein array system (Bio-Rad) using Bio-Plex Manager 3 software.

***In vitro* assessment of macrophage cytokine secretion**

Macrophages were plated at a density of 0.5-0.75 million cells per well of a 24 well plate. The following day, cells were stimulated for the indicated time points with LPS (1 μg/ml). TNF and IL-6 ELISAs were performed according to the manufacturer's instructions (BD Bioscience).

**Flow cytometry**

Macrophages were treated for 3 hours with or without 10 μg/ml LPS. Cell suspensions ($1x10^6$ cells) were stained for 30 minutes at 4°C with CD11b-PE (eBioscience) and anti-TNF-APC (BD Bioscience) antibodies in PBS containing 3% FCS; washed twice and analysed on FACS Calibur (BD Bioscience). After gating on CD11b positive cells, surface TNF levels were quantified using FlowJo 2.

**Cell Surface Biotinylation**

2-4 x $10^7$ BMDMs were washed 3 times in ice-cold PBS. Where indicated, cells has been pretreated for 3 hours with 1μg/ml LPS. Subsequently surface proteins were labelled with 1 mg/ml Sulfo-NHS-LC-Biotin (Thermo Scientific, 21335) according to

the manufacturer's instructions. Cells were lysed on ice for 10 minutes in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 containing complete protease inhibitor cocktail (Roche) and 10 mM 1,10-phenanthroline (Sigma)) and spun at 16,000g at 4°C to obtain post-nuclear supernatants. Biotin labelled surface proteins were captured on neutravidin agarose resin (Thermo Scientific, 29200) at 4°C overnight. The resin was washed 3 times with lysis buffer containing 300 mM NaCl. Proteins bound to the resin were eluted with 1x SDS PAGE sample buffer, and analysed by western blotting. To enhance the detection of ADAM10 in macrophage lysates, lysates were subjected to an affinity purification step using ConA-agarose (Sigma) in standard lysis buffer supplemented with 1 mM each of $CaCl_2$, $MnCl_2$ and EDTA.

**Metabolic Labelling**

Macrophages were washed once in PBS prior to amino acid starvation for 25 minutes in methionine/cysteine-free DMEM (Gibco) containing dialyzed, amino acid-free FCS and supplemented with 10 mM HEPES pH 7.4. Cells were radiolabelled for 3h with $^{35}S$ methionine/cysteine (Easytag Express protein labelling mix, Perkin Elmer), after which they were washed twice in ice-cold PBS, lysed as described above and subject to immunoprecipitation using chicken anti-TACE or control IgY antibodies.

**Deglycosylation analysis**

Macrophage cell lysates were generated as described above for the cell surface biotinylation. Lysates were then denatured at 90$^{o}$C and treated with endoglycosidase H or PNGase F according to the manufacturer's instructions (New England Biolabs). Note that for all assays requiring western blot analysis, lysis buffer was supplemented with 10 mM 1,10-phenanthroline to prevent autoproteolysis of TACE (*18*).

**In vitro TACE enzymatic assay**

2-4 x $10^7$ macrophages were lysed for 10 minutes on ice in 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 containing complete protease inhibitor cocktail (Roche), without 1,10-phenanthroline, to preserve TACE activity. Chicken anti-TACE and chicken IgYs covalently coupled to CNBr activated Sepharose 4B (Sigma, C9142) were used in immunoprecipitations. Chicken anti-TACE and chicken IgY covalently coupled to CNBr activated Sepharose 4B (Sigma, C9142) were used in immunoprecipitations. IPs were washed three times in lysis buffer and subsequently equilibrated in assay buffer (ANASPEC, 72085). Immunoprecipitates were mixed with the fluorogenic TACE substrate peptide and fluorescence was measured over 3 hours in a Pherastar 96 well plate reader according to manufactures instructions (ANASPEC SensoLyte 520 TACE Activity Assay Kit, 72085). For the experiment in Fig 4H, where exogenous furin was used to rescue TACE activity, immunoprecipitates were washed three times in lysis buffer and twice in furin assay buffer (35 mM Tris, pH 7.4, 1 mM CaCl2, 0.5% TX-100) prior to incubation in 100 nM recombinant mouse furin (R & D Systems, 6450-SE-010) for 1 hour at 37$^o$C in furin assay buffer. TACE activity was then measured as described above. Upon cell lysis, active TACE removes its own cytoplasmic tail by autocatalysis (*18*). However, the chicken anti-TACE antibody used in the activity assay immunoprecipitates recognizes an epitope within the ectodomain and captures TACE regardless of autocatalysis; mature TACE without its cytoplasmic domain retains proteolytic activity (our data and Carl Blobel, personal communication). Note however that the rabbit polyclonal antibody used for western blotting recognizes an epitope within the TACE tail; consequently, lysates generated in the absence of 1,10-phenanthroline show no evidence of mature TACE (Fig 3E, right hand side of panel; inputs on the left hand were generated from parallel lysates containing 1,10-phenantholine).

**Lentiviral transduction of 293ET**

Lentivirus was produced as described previously (*24*). 293ET cells were transduced with either pLEX.puro empty vector or pLEX.puro containing mouse iRhom2 with a C-terminal HA tag and were selected in 5 μg/ml puromycin.

**Co-immunoprecipitations**

293ET cells stably expressing vector or iRhom2-HA were lysed directly for immunoprecipitation (Fig 4D, 4F and S3B,C). Alternatively, they were first transfected using Fugene 6 (Roche) with C-terminal V5-tagged versions of mouse ADAM17 or mouse ADAM10 (*25*) (Fig 4A), or C-terminally Strep-tagged TACE (Fig S3A). 20-24 hours post-transfection cells were washed twice with ice-cold PBS and incubated for 10 minutes on ice in TX-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing complete protease inhibitor cocktail (Roche) plus 10 mM 1,10-phenanthroline. Where indicated, RIPA buffer (TX-100 buffer supplemented with 0.5% sodium deoxycholate and 0.1% SDS) was substituted instead of TX-100 buffer. The post-nuclear supernatants were pre-cleared by incubating with mouse IgG-agarose or with rabbit IgG-agarose (Sigma) for one hour at 4°C. Immunoprecipitations were performed using anti-HA or anti-V5 affinity resins (Sigma), or anti-TACE rabbit polyclonal plus protein A resin (GE Healthcare) for 1-2h at 4°C. Beads were washed extensively at room temperature 4-5 times (10 minutes per individual wash) in TX-100 or RIPA buffer supplemented to 300 mM NaCl, before eluting into 1x SDS-PAGE loading buffer. Alternatively, samples were eluted into 1 mg/ml HA peptide (Roche) in wash buffer (Fig. S3A,B). SDS PAGE sample buffer containing 50 mM DTT was then added and samples were reduced for 15 minutes at 65°C prior to SDS-PAGE and immunoblotting.

For endogenous IPs from macrophages, RIPA lysates were precleared for 1 hour in

chicken IgY-sepharose (Fig 4B) or rabbit IgG-agarose (Fig 4C), after which they were immunoprecipitated for 1-2 hours with IgY-sepharose, or chicken anti-TACE-sepharose (Fig 4B), or rabbit pre-immune, or anti-iRhom2 antibodies plus protein A resin (Fig 4C). IPs were washed stringently: 5 times in RIPA buffer (10 minutes each at room temperature) prior to elution with SDS-PAGE lysis buffer. Note that to avoid cross-reactivity with the rabbit IP antibodies in Fig 4C, HRP-conjugated iRhom2 and TACE rabbit polyclonal antibodies were used for western blotting detection.

**In vivo crosslinking of TACE and iRhom**

Cells were washed twice in ice cold PBS, after which they were incubated for 45 minutes in 0.2 mg/ml DSP (Dithiobis (succinimidyl proprionate); Thermo Scientific). The cross-linker was removed and cells were washed three times in ice-cold PBS containing 50 mM Tris, pH 8.0, to quench any remaining crosslinker. Cells were then lysed and used for immunoprecipitations as described above.

**siRNA mediated knockdown of iRhoms**

HeLa or HEK cells were transfected with ON-Target plus siRNA smartpools (Thermo Scientific) targeting human iRhom1/RHBDF1 (L-015651-02), human iRhom2/RHBDF2 (L-015783-00). An irrelevant oligo against human RHBDL2 (L-006028-00) was used as a negative control. Cells were transfected in six-well plates with 300 pmoles of siRNAs, using 15 µl of oligofectamine (Invitrogen). After 24 hours, cells were transfected identically a second time, and after 48 hours they were transferred into 10 cm plates. 72 hours after the first transfection, cells were lysed in lysis buffer supplemented with 1-10, phenanthroline, as described above. The ability of the oligos to knockdown expression was confirmed using qPCR and the indicated probes: (iRhom1/RHBDF1, Hs01102745_m1; iRhom2/RHBDF2, Hs00226277_m1; RHBDL2, Hs00384848_m1, Applied Biosystems).

## 2. Supplemental figure legends

**Figure S1.** Generation of iRhom2 mutant mice. **(A)** Map of the mouse *iRhom2* genomic locus comprising exons 3-10 (top). The targeting vector introduces loxP sites (red arrow heads) flanking exons 4-9 (grey bars) and a PGK Neo cassette flanked by FRT sites (white arrow heads) into the chromosome of ES by homologous recombination. Site specific targeting events were enriched by positive (Neo) and negative (TK) selection. The targeted iRhom2 locus harbours an extra BamHI site resulting in a 6 kbp fragment as opposed to the 7.8 kbp WT allele when detected with a 3' flanking probe (indicated by a red line). Genomic DNA comprising exons 4-9 was excised by cre expression in ES cells (excised regions indicated in white). (**B**) Southern blot of BamHI-digested genomic DNA from WT and heterozygous (Het) ES cells showing an additional 6 kbp fragment (indicated by an arrow) in the Het ES cells demonstrating site specific integration of the targeting construct. Note the probe (red bar) was designed to anneal outside of the 3' homology arm. (**C**) Genotyping PCR using genomic DNA from WT, iRhom2 KO and Het mice using primers x, y and z (see A). Primers x and y amplify a 230 bp fragment only in WT animals, whereas primers x and z detect a 500 bp fragment only in the KO. (**D**) iRhom2 mRNA expression monitored by RT-PCR using combinations of primers a, b, c and d (see panel A). The lack of amplification in iRhom2 KO cells using primer set a+b and the amplification of products using primers a+c and a+d indicates successful excision of exons 3-9 and confirms the targeting strategy.

**Figure S2.** TACE immunoprecipitation and trafficking controls. (**A**) Induction of TNF mRNA is normal in KO macrophages. WT or KO macrophages were treated

with LPS (1 μg/ml) for the indicated time points, after which RNA was harvested and cDNA generated. qPCR was performed and TNF mRNA levels are expressed as a fraction of the control mRNA for TBP (see Methods). (**B**) Untreated WT or iRhom2 KO BMDMs were radiolabelled for 3h with 35S methionine/cysteine, and anti-TACE immunoprecipitates were detected by SDS-PAGE/autoradiography. Full length TACE is indicated with an arrowhead; the asterisk denotes a TACE degradation product. This result confirms that equal amounts of TACE were assayed in the activity measurement from WT and KO macrophages. (**C**) Schematic diagram explaining molecular weight changes (MW) of TACE during trafficking through the secretory pathway, and its behaviour in response to deglycosylating enzymes. TACE is synthesized in the ER as an immature precursor (a) containing ER N-glycans. These can be experimentally removed by endo-H to generate a polypeptide with an lower apparent molecular weight (b). Upon entry into the Golgi apparatus, TACE acquires elaborated N-glycans, becoming endo-H-resistant. This intermediate cannot normally be detected because furin rapidly cleaves off the inhibitory N-terminal prodomain; however, this can be revealed by inhibiting furin (see band c). Furin cleavage generates a species (d) that in mouse macrophages has an apparently similar mobility to immature TACE (a); in untreated extracts these co-migrate. However, when treated with endo-H, the endo-H-insensitive furin-cleaved species (d) represents a smear that migrates more slowly than endo-H-sensitive, immature TACE (a; see also Figure 3C where the gel is better resolved). When mature TACE (d) is treated with PNGase F, the resulting polypeptide has a much lower apparent molecular weight (e) than deglycosylated immature TACE (b). (**D**) Inhibition of furin causes the accumulation of an immature TACE intermediate containing the prodomain with endo-H resistant, Golgi N-glycans (black arrowhead ‘c’). Note that this species is not normally detected

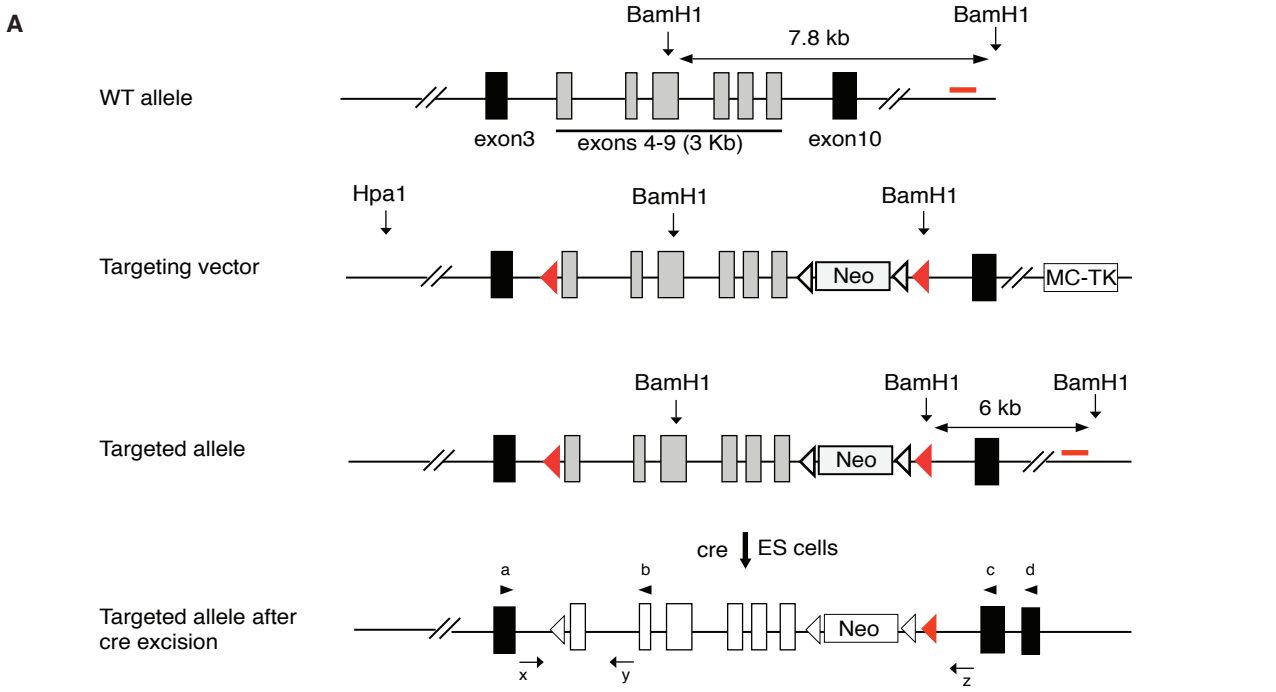
when furin is active because of rapid furin-mediated proteolysis. Furin inhibition also reduces the generation of the mature form lacking the prodomain, confirming the origin of this band (white arrowhead 'e'). Individual TACE intermediates described in (C) are labelled on this western.

**Figure S3.** (**A**) iRhom2 binds TACE specifically. Immunoprecipitates from HEK cells stably expressing iRhom2-HA were transfected with mouse TACE-strep or mouse ADAM10-V5. Immunoprecipitated TACE-strep or endogenous TACE are indicated by black or white arrows respectively. No immunoprecipitation of ADAM10 was detected. iRhom2-HA levels were determined by anti-HA western Blot. (**B**) iRhom2 specifically immunoprecipitates endogenous TACE. Stable vector or iRhom2-HA expressing 293ET cells were lysed and iRhom2 was captured in anti-HA immunoprecipitates. Lysates versus immunoprecipitates were immunoblotted for the indicated endogenous proteins, including TACE (white arrow). iRhom2 was unable to immunoprecipitate the transferrin receptor (TfnR), cadherin, EGF receptor or PERK. (**C**) Reciprocal immunoprecipitation of endogenous TACE with iRhom2-HA. Stable vector or iRhom2-HA expressing 293ET cells were lysed and TACE was captured in TACE immunoprecipitates. Lysates versus immunoprecipitates were immunoblotted for TACE or with HA-HRPO to detect iRhom2-HA.

### 3. Supplemental references


23. P. Liu, N. A. Jenkins, N. G. Copeland, *Genome Res.* **13**, 476 (2003).
24. C. Adrain, K. Strisovsky, M. Zettl, L. Hu, M. K. Lemberg, M. Freeman, *EMBO Rep.* **12**, 421 (2011).
25. S. Lammich, D. Buell, S. Zilow, A. K. Ludwig, B. Nuscher, S. F. Lichtenthaler, C. Prinzen, F. Fahrenholz, C. Haass, *J. Biol. Chem.* **285**, 15753 (2010).

Figure S1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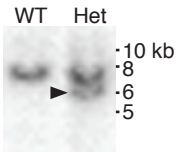
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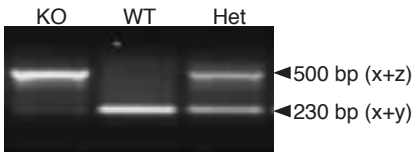
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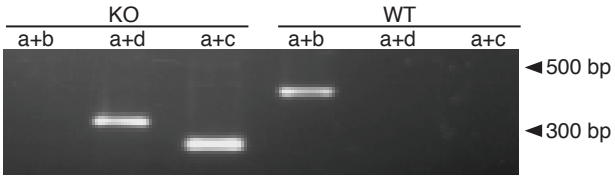
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Figure S2

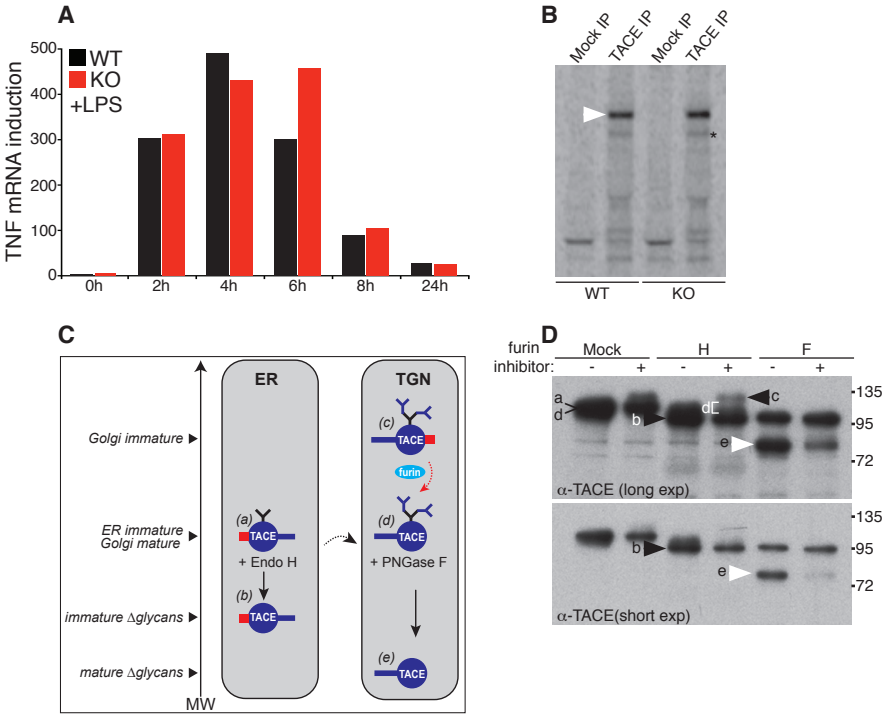
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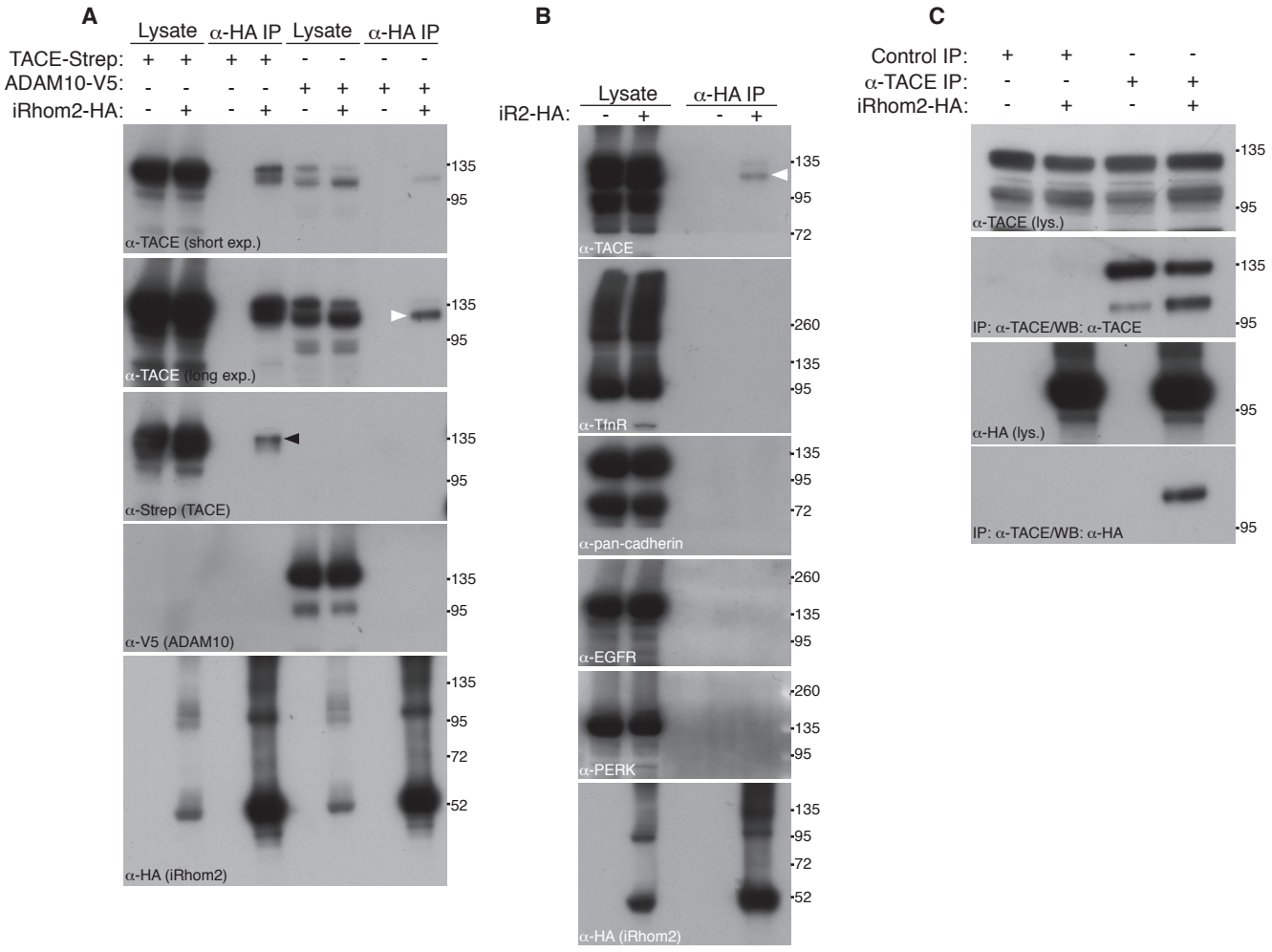
Figure S3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