Deletions in the cytoplasmic domain of iRhom1 and iRhom2 promote shedding of the TNF receptor by the protease ADAM17[†]

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Abstract:

The protease ADAM17 catalyzes the shedding of various transmembrane proteins from the surface of cells, including tumor necrosis factor (TNF) and its receptors. Liberation of TNF receptors from cell surfaces can dampen the cellular response to TNF, a cytokine that is critical in the innate immune response and promotes programmed cell death but can also promote sepsis. Catalytically inactive members of the Rhomboid family of proteases, iRhom1 and iRhom2, mediate the intracellular transport and maturation of ADAM17. Using a genetic screen, we found that presence of either iRhom1 or iRhom2 lacking part of their extended N-terminal cytoplasmic domain (herein referred to as ΔN) increases ADAM17 activity, TNF receptor shedding, and resistance to TNF-induced cell death in mouse embryonic fibroblasts and fibrosarcoma cells. Inhibitors of ADAM17, but not of other ADAM family members, prevented the effects of iRhom-ΔN expression. iRhom1 and iRhom2 were functionally redundant, suggesting a conserved role for the iRhom N-termini. Cells from patients with a dominantly inherited cancer susceptibility syndrome called tylosis with esophageal cancer (TOC) have N-terminal mutations in iRhom2. Keratinocytes from TOC patients exhibited increased TNFR shedding compared with cells from healthy donors. Our results explain how loss of the N-terminus in iRhom1 and iRhom2 impairs TNF signaling, despite enhancing ADAM17 activity, and may explain how mutations in the N-terminal region contribute to the cancer predisposition syndrome TOC.

Introduction:

A disintergrin and metalloproteinase 17 (ADAM17) [also known as TNFα converting enzyme (TACE)] is a membrane-anchored metalloproteinase, capable of processing a wide array of cell surface/membrane proteins, and is a central regulator of epidermal growth factor receptor (EGFR) and tumor necrosis factor receptor (TNFR) signaling pathways, which control cell proliferation, survival, oncogenesis and immunity(*1*). TNF is liberated from its membrane anchor by ADAM17 to produce a soluble pro-inflammatory cytokine(*2-4*). However, ADAM17 can also modulate responses to this cytokine by catalyzing shedding of TNF-binding receptors p55 (TNFR1) and p75 (TNFR2)(*5, 6*). TNFR1 signaling is a key component of innate immunity, host defense, and septic shock(*7, 8*), yet TNFR1 engagement can also induce cell death through signaling leading to activation of caspase-8(*9*).

Recently, ADAM17 was identified to be controlled by catalytically inactive members of the rhomboid protease family: iRhom1 and iRhom2. These integral membrane proteins promote the maturation and transport of ADAM17 to the cell surface(*10-13*). Absence of iRhom2 abolishes ADAM17 activity in immune cells thereby blocking TNF secretion, resulting in susceptibility towards bacterial infections but resistance to septic shock and rheumatoid arthritis (*11-14*). In non-hematopoietic cells ADAM17 appears to be controlled by a combination of iRhom2 and iRhom1(*10*). The essential role of iRhoms in regulating the function of ADAM17 are highlighted by recent iRhom1 and iRhom2 double knockout studies demonstrating fully impaired ADAM17 maturation across all tissues examined (*15*) and striking similarity between *iRhom1*$^{-/-}$*iRhom2*$^{-/-}$ mice and ADAM17 deficient mice(*15*). However, much remains to be learned about how iRhoms accomplish their regulation of ADAM17, and what features of iRhoms are important for their function.

Recent reports have identified familial dominantly acting mutations in the N-terminal cytoplasmic tail of iRhom2 (also known as RHBDF2 in humans) as causative for a rare syndrome named tylosis with oesophageal cancer (TOC), which is characterized by palmoplantar keratoderma and up to 95% lifetime risk of malignancy of the oesophagus (*16, 17*). These TOC-associated mutations in iRhom2 are associated with an increase in the maturation and activity of ADAM17 in patient-derived epidermal keratinocytes, resulting in significantly upregulated shedding of ADAM17 substrates, including EGF-family growth factors and pro-inflammatory cytokines (*18, 19*). Furthermore, studies of two separate spontaneous mouse mutants (*cub* and *uncv*) reveal hair and skin abnormalities also associated with deletions in the N-terminus of iRhom2(*11-14, 20*). Although the immediate consequences of these murine mutations are not entirely clear especially in the case of *cub* mice, all studies in both humans and mice suggest phenotypes involving misregulation of ADAM17 substrates, and offer a clue that the N-terminal domain of iRhom2 may be important for controlling its activity(*21*).

Interestingly, our initial identification of a connection between iRhom2 and ADAM17 involved a cyclic packaging rescue (CPR) screen for TNF resistance, which identified a version of iRhom2 with a truncated N-terminus(*12*). Here we report a separate TNF resistance CPR screen which identified two versions of iRhom1, both also missing parts of their N-termini. In order to gain more insight into how iRhoms operate, we examined the ability of truncated and full length iRhoms to regulate ADAM17 activity in a well-defined cellular context. We observed a high degree of functional overlap for iRhom1 and iRhom2, and demonstrate that deletion of parts of the cytoplasmic N-terminus of iRhom2 or iRhom1 results in specific enhancement of ADAM17

activity, TNFR shedding, and resistance to TNF-induced cell death. Our results support the link of N-terminal iRhom mutants with constitutive activity of ADAM17.

Results:

Truncation of iRhom2 or iRhom1 cytoplasmic domains triggers resistance against TNF-induced cell death

L-929 murine fibrosarcoma cells are highly sensitive to TNF-induced cell death through engagement of their cognate cell surface receptors(*22-24*). cDNAs capable of conferring resistance to L-929 cell killing by TNF were identified from a mouse 3T3 cell-derived cDNA library through enrichment in a CPR screen(*25*). Three different cDNAs were isolated following six successive rounds of infection, cell killing, and rescue of viral particles from surviving cells (Fig. 1A). Sequencing revealed the identity of these hits as c-FLIP, an established negative regulator of TNF induced cell death(*26*), along with two cDNAs corresponding to nucleotides 249-2571 and 618-2571 of native iRhom1 (the latter referred to henceforth as iRhom1-ΔN) (Fig. 1B and fig. S1A). The similarity of this result to the identification of an N-terminally truncated version of iRhom2 we previously reported using a separate CPR screen(*12*) and recent literature concerning mutations in the N-terminus of iRhom2(*20, 21, 27*) led us to investigate whether there was a selective advantage for removal of part of the extended cytoplasmic N-terminus, a hallmark feature of iRhoms(*28*).

Full-length and ΔN truncated iRhom proteins were detected at predicted molecular weights, and in similar abundance, in cells stably overexpressing tagged iRhom constructs (Fig. 1C). We first compared the ability of cells overexpressing either full-length iRhom2-WT,

iRhom2-ΔN or vector control to withstand exposure to recombinant TNF (Fig. 1D). We observed only a slight reduction in cell death assessed by Annexin V and 7AAD staining for cells expressing iRhom2-WT, whereas iRhom2-ΔN cells had dramatically lower levels of Annexin V, 7AAD positive cells (Fig. 1D). Interestingly, we found similar effects when comparing cells expressing iRhom1-WT versus iRhom1-ΔN, which were also strongly resistant to TNF cytotoxicity (Fig. 1E). Next, we confirmed these observations using a separate assay to measure cells remaining adherent following 48 hours of TNF treatment. Consistently, we observed a slight protection against TNF mediated cell death in iRhom2-WT expressing cells, but major protection was triggered by iRhom2-ΔN (fig. S1B). Moreover, iRhom1-ΔN expressing cells were also protected against TNF induced cell death, compared to iRhom1-WT (fig. S1C). Importantly, cells expressing full-length and ΔN iRhoms retained a similar capacity to undergo cell death resulting from other stimuli, such as staurosporine (fig. S1D). These data indicate that deletion of part of the cytoplasmic domain of either iRhom1 or iRhom2 confers a selective advantage over their full length counterparts in TNF resistance.

Truncated iRhoms curb TNFR signaling through release of TNF receptors

We next investigated the mechanism by which iRhom2-ΔN confers resistance towards TNF-induced cytotoxicity by examining the status of signaling downstream of the TNFRs. TNFR engagement in L-929 cells results in activation of both cell survival and cell death signaling pathways, causing activation of cell survival associated NF-κB and cleavage of cell death associated poly(ADP ribose) polymerase (PARP) (*29-32*). When we examined PARP levels by immunoblot, we detected cleaved PARP in TNF-treated control cells (Fig. 2A). However, PARP cleavage was reduced in cells expressing iRhom2-WT, and was not detected in

cells expressing iRhom2-ΔN (Fig. 2A). Next, we looked for hallmarks of TNF-mediated NF-κB activation(*33*) and detected IκB-α phosphorylation and degradation as well as serine$^{(536)}$ phosphorylation of p65 in vector and iRhom2-WT expressing cells, but not in iRhom2-ΔN expressing cells (Fig. 2B). These findings indicate that TNFR signaling was blocked by iRhom2-ΔN upstream of both survival and death signaling branches(*9*).

To test whether surface TNFR abundance itself was affected by different iRhom2 isoforms, we used flow cytometry to measure TNFR abundance on the surface of L-929 cells transduced with vector, iRhom2-WT, or iRhom2-ΔN. TNFR1 and TNFR2 abundance was highly reduced on cells expressing iRhom2-ΔN or iRhom1-ΔN compared to cells expressing vector or either wild-type iRhom (Fig. 2, C and D). When we measured the concentration of soluble TNFRs in conditioned media from these cultures, we observed greater concentrations of soluble TNFR1 and TNFR2 in conditioned media from cells expressing iRhom2-ΔN or iRhom1-ΔN than in that from control cells or cells expressing either wild-type iRhom (Fig. 2, E and F). These effects were not associated with either decreased *TNFR* transcript expression (fig. S2A+B) or large differences in total cellular abundance of TNFRs (fig. S2, C-E).

Together, the data indicate that the blockade in TNFR signaling after ΔN-iRhom expression was caused by reduced surface abundance and enhanced shedding of TNFRs. Previous work suggested that shedding of TNFRs was responsible for iRhom2-mediated resistance towards TNF by blocking TNFR signaling(*11, 12*). We now show that this shedding mechanism is not only true for truncated iRhom2 but also for truncated iRhom1. Furthermore, we confirm that TNFR signaling is blocked by ΔN-iRhom expression by showing that both downstream cell survival and cell death signaling responses are impaired in these cells.

iRhom-regulated TNFR shedding depends on ADAM17

TNFRs are cleaved from the cell surface by membrane proteases such as ADAM17 (*5, 6*) (fig. S3A). To investigate whether increased TNFR shedding into the supernatant of iRhom-ΔN expressing cells was dependent on metalloproteases in L-929 cells, we investigated shedding in the presence of several ADAM family inhibitors. Increased release of TNFRs into the supernatant of iRhom2-ΔN expressing cells relative to control or wild-type iRhom2 was blocked by the broad spectrum metalloprotease inhibitor marimastat (Fig. 3A). Furthermore, shedding induced by iRhom2-ΔN or iRhom1-ΔN was unaffected by an ADAM10 selective inhibitor, GI254023X (GI) (*34*), but was abolished by an inhibitor of both ADAM10 and ADAM17, GW280264X (GW) (Fig. 3, B and C). Consistently, culturing iRhom2-ΔN expressing cells with marimastat restored the ability of recombinant TNF to trigger cell death to a similar extent as that observed in cells expressing wild-type iRhom2 (Fig. 3D). TNF itself had little effect on TNFR shedding (fig. S3, B and C).

To firmly establish that ΔN-iRhoms exert their effects through ADAM17, we stably expressed either a scrambled control or one of two ADAM17-specific shRNAs (Fig. 4A) in L-929 cells transduced with vector, iRhom2-WT, and iRhom2-ΔN. Surface TNFR1 and TNFR2 abundance was reduced in cells expressing iRhom2-ΔN or iRhom1-ΔN relative to wild-type or vector-transduced controls, however these effects were rescued by ADAM17 silencing (Fig. 4B and fig. S4, A and B), suggesting that truncated iRhoms enhance ADAM17-dependent shedding. Next we examined whether ADAM17 silencing would disrupt the survival advantage of ΔN-

iRhoms against TNF. As expected, knocking down ADAM17 abolished the survival advantage caused by expression of ΔN-iRhoms conferred in response to TNF (Fig. 4, C and D).

Together, the data indicate that TNFR shedding and TNF resistance phenotypes associated with expression of N-terminally truncated iRhoms can be blocked by knockdown or inhibition of ADAM17, establishing ADAM17 as a mechanism through which iRhoms mediate their effects on TNF signaling, at least in the cell context studied.

Truncation of the cytoplasmatic tail results in enhanced presence of iRhom2 at the cell surface

We next asked how wild-type iRhoms and their ΔN counterparts might differentially affect ADAM17 activity. Binding of iRhom2 to ADAM17 is thought to be important for ADAM17 maturation and activation (*11-13*). When we pulled down either wild-type or truncated iRhom2, we detected ADAM17 in both immunoprecipitated lysates (Fig. 5A). Reciprocally, when ADAM17 was pulled down, both iRhom2-WT and iRhom2-ΔN were detected (Fig. 5A). Similar data was obtained in iRhom1-WT and iRhom1-ΔN expressing cells (fig. S5A). These data indicated that both isoforms of iRhom1 and iRhom2 are capable of binding ADAM17, consistent with previous reports (*12, 35*).

When we enriched for cell surface proteins, we noticed a larger proportion of iRhom2-ΔN was present in surface fractions versus intracellular fractions and relative to wild-type iRhom2 (Fig. 5B). These findings were further confirmed by T7 surface antibody staining and flow cytometry, where the MFI for iRhom2-ΔN was significantly higher than that for wild-type iRhom2 (Fig. 5C). Consistently, immunofluorescence of tagged iRhom2 revealed greater staining intensity for iRhom2-ΔN versus wild-type iRhom2 on formalin fixed cells, differences

which were not apparent after 1% triton permeabilization (Fig. 5D). Furthermore, in permeabilized cells, sub-cellular localization of both wild-type and ΔN-iRhoms in the proximity of Golgi marker GM130 (fig. S5B) was consistent with previous reports (*12, 36*). Increased surface localization of iRhom2-ΔN compared with wild-type iRhom2 persisted after ADAM17 knockdown and in ADAM17 knockout fibroblasts (Fig 5, E and F)(*4*), indicating that ADAM17 is not strictly required for iRhom2-ΔN trafficking.

Truncated iRhom2 and wild-type Rhom2 did not exhibit significantly different rates of protein degradation after inhibition of protein synthesis with cycloheximide, as measured by immunoblotting (fig. S5C). This argues against a role for differential protein stability affecting iRhom2 surface abundance. Increased surface abundance of iRhom2-ΔN is also unlikely to be the result of greater RNA expression, as transcript abundance was comparable between iRhom2-ΔN and wild-type iRhom2 (fig. S5D). We detected mature ADAM17 on the cell surface of cells expressing vector, wild-type iRhom2, or iRhom2-ΔN (fig. S5E). However, a puzzling overall reduction in the total quantity of mature ADAM17 was observed in immunoblots of cells expressing iRhom-ΔN (Fig. 5A and fig. S5, A and E), an effect that was prevented by marimastat treatment (fig. S5F). These data indicate that ΔN-iRhoms are capable of binding to ADAM17 in a similar manner to that of wild-type iRhoms, and that the specific effects of iRhom2-ΔN may be related to its enhanced abundance at the cell surface.

N-terminal iRhom mutations increase constitutive activity of ADAM17

ADAM17 can be rapidly activated in response to stimuli including PMA (*37, 38*). We therefore wondered whether the effects of ΔN-iRhoms might influence the rapid activation of ADAM17. When we stimulated L-929 cells with PMA, we observed an expected increase in TNFR1 and

TNFR2 shedding from cells expressing either vector or wild-type iRhom2 (fig. S6A). In contrast, we did not detect PMA-induced TNFR shedding from cells expressing iRhom2-ΔN (fig. S6A), and the amount of TNFRs in the stimulated cells expressing iRhom2-ΔN was not increased when compared to cells expressing wild-type iRhom2 (fig. S6A) In all cases, PMA-stimulated TNFR shedding was blocked by marimastat (fig. S6A), or GW, which both inhibit ADAM17, but not by the ADAM10-selective inhibitor GI (Fig. 6A). Furthermore, similar data using cells expressing iRhom1-ΔN or wild-type iRhom1 indicated that both truncated iRhoms may induce a constitutively active TNFR shedding state, resembling PMA-stimulated shedding state in control cells (Fig. 6B).

To investigate these effects in a different cellular context we co-expressed wild-type or truncated iRhom2 or as control, an unrelated cytoplasmic protein MAD2 (mitotic arrest deficient 2) along with the iRhom2-selective AP-tagged ADAM17 substrate KitL2 in *iRhom2*$^{-/-}$ immortalized mouse embryonic fibroblasts (iMEFs) (*10, 12*). To improve the detection of potential differences in shedding, we took advantage of the reversible nature of ADAM17 inhibition with marimastat to block shedding overnight, enabling uncleaved substrate to accumulate, and then observed constitutive shedding immediately after washout of the inhibitor. Compared to control cells, only cells expressing iRhom2-ΔN exposed overnight to marimastat exhibited greatly enhanced shedding of KitL2 (Fig 6C). In a separate experiment, we used an irreversible inhibitor of ADAM17 (DPC), which selectively binds to active ADAM17. Thus, if ADAM17 is not activated while cells are exposed to DPC, ADAM17 can be fully activated after the inhibitor has been removed; however, if ADAM17 is activated in the presence of DPC, ADAM17 will not be able to recover activity after the inhibitor is removed (*37*). Incubation of cells expressing iRhom2-ΔN with DPC for as little as 30 minutes prevented the increased KitL2

shedding even after the inhibitor was removed, whereas cells expressing wild-type iRhom2 required overnight exposure to DPC to exhibit ADAM17 inactivation (fig. S6B). These data suggest that ADAM17 is constitutively in a more active state in the iRhom2-ΔN setting.

Dominantly acting familial mutations in the N-terminal cytoplasmatic tail of iRhom2 cause a cancer susceptibility disorder called tylosis with esophageal cancer (TOC) (*16, 17*). These mutations increase constitutive ADAM17 activity in keratinocytes (*18*). Compared to overexpression of wild-type murine iRhom2 in iRhom1 and 2 double knockout iMEFs, expression of a construct bearing mouse homologues of two causative iRhom2$^{I156T/P159L}$ TOC mutations caused increased constitutive TGF-α shedding (Fig 6D). Finally, we examined human immortalized keratinocyte cell lines TYLK 1 and TYLK2 derived from TOC patients bearing heterozygous mutation in iRHOM2 $^{(I186T/WT)}$ (*16, 17*) and control keratinocyte cell lines (K17). Significantly higher amounts of constitutive TNFR1 shedding into culture supernatants was detected from cells generated from TOC patients compared to cells derived from healthy controls (Fig 6E), shedding which could be blocked by the ADAM17 inhibitor TMI-005. This data is consistent with enhanced ADAM17-dependent shedding of amphiregulin, TGF-α and HB-EGF that is observed in TOC patient-derived keratinocytes (*18*)

These results indicate that ADAM17 activity is enhanced by N-terminal iRhom mutations and may provide an explanation for why cells that have ΔN-iRhom proteins have a selective advantage over their wild-type counterparts in promoting resistance to TNF-induced cell death.

Discussion:

In this study we have identified a high degree of functional overlap for iRhom1 and iRhom2 in controlling the activity of ADAM17. Starting from unbiased genetic screens we have established that deletion of part of the cytoplasmic N-terminus of either iRhom1 or iRhom2 results in a gain of function by promoting constitutive ADAM17 dependent shedding of TNF receptors from L-929 cells, thereby blocking downstream signaling, and protecting cells from TNF induced cytotoxicity. We demonstrate that iRhom1 and iRhom2 dependent effects are conserved for at least two endogenous ADAM17 substrates (TNFR1 and TNFR2), and using gene silencing show these effects to be specific to ADAM17.

Little is known about how the iRhoms are functionally controlled. Our results suggest that the extended cytoplasmic N-terminal domain is of conserved importance for the actions of both iRhom1 and iRhom2. Dominant inherited human N-terminal mutations in iRhom2 result in overgrowth of epithelial tissues and predisposition to oesophageal cancer (*39*). Our results involving dominant N-terminal mutated mouse iRhoms and enhanced TNFR shedding from TOC-derived keratinocytes suggest that these human mutations could similarly result in greater or constitutive ADAM17 activity. This could result in direct consequences for neoplasia, such as over production of EGFR ligands, strongly implicated in cancer (*40*) and previously observed in TOC keratinocytes (*18*), or shedding of TNFRs as an evasion mechanism for tumor cells to escape cell death induced by TNF. Increased abundance of ADAM17 is also known to be associated with breast, ovarian, kidney, colon and prostate cancers. (*41*)

Recent studies involving two separate spontaneous mouse mutants with deletions in the N-terminus of iRhom2 describe hair and skin abnormalities in these animals(*20, 21, 27, 42*). Discerning the consequences of these mutations has not been entirely straightforward; Hosur et al. report enhanced stability of mutant iRhom2 in *cub* mice and elevated secretion of ADAM17

substrate amphiregulin, observations not corroborated by Siggs et al. (*20*) in the same mice. Mice bearing a separate *Uncv* allele display aberrancies in hair follicles, which may be connected to misregulation of ADAM17 substrates. In the case of both *cub* and *Uncv,* expression of mutant alleles results in an apparent decrease in mature ADAM17 by Western blotting (*20, 27*). Paradoxically, we also see an apparent reduction in mature ADAM17 by immunoblot following expression of ΔN-iRhoms, despite strong evidence of enhanced ADAM17 activity. Since this effect could be reversed by inhibiting ADAM17 with marimastat, we hypothesize that the apparent reduction of mature ADAM17 may be an artifact of activation-associated autocatalytic degradation of ADAM17 known to occur when it is strongly activated, such as by high concentration of PMA (*43*). Rapid inhibition of ADAM17 activity with DPC in iRhom2-ΔN expressing cells provides further evidence for enhanced constitutive ADAM17 activity in this context.

Aside from the mouse and human mutations listed above, the in vivo relevance of alternative iRhom1 or iRhom2 isoforms has not yet been described. However, expression of C-terminally tagged iRhom2 and iRhom1 frequently results in lower molecular weight bands, which could correspond to post-translationally processed iRhoms lacking N-terminal regions (*11, 44*). Whether these versions exist for endogenous proteins, and whether they represent activated iRhom states, warrants investigation in future studies, including the generation of high quality antibodies. Interestingly, similar to our results, a previous study involving expression of human iRhom1 (*44*) (then referred to as p100hRho) in the fly wing identified a phenotype for an N-terminally truncated, but not full-length version of the protein. Because iRhom1 has been previously suggested to play a role in human cancer (*45, 46*), and considering functional overlap,

we have identified between N-terminal mutations in iRhom1 and iRhom2, the potential existence of human mutations in iRhom1 and their contributions to cancer warrants further study.

Materials and Methods:

CPR screening: L-929 selector cell line was established by transfection of expression plasmids coding for mCAT1 (murine cationic amino acid transporter, mediating ecotropic retrovirus infection) and hCAR (human coxsackievirus and adenovirus receptor) and selected for high infectivity to GFP-expressing ecotropic retrovirus and human adenovirus, and high susceptibility to TNF. CPR was carried out as described previously (*25*). The Phoenix ecotropic packaging cell line was transfected with retroviral mouse 3T3 cell cDNA library by using calcium phosphate coprecipitation. Retroviral supernatants were obtained 2 days after transduction and used to transduce the selector cells. Two days after retrovirus transduction, cells were cultured with TNF (2 ng/ml) for 24 hours. Surviving cells were allowed to recover from TNF stress and expanded in culture for 3 days, then infected with adenoviruses expressing gag-pol and env (amounts of adenoviruses were determined empirically). Rescued retroviruses were harvested 2 days after adenovirus infection and used to transduce new batches of selector cells. Genomic DNA was extracted from a fraction of the surviving cells at each round of screening. A portion of cDNA inserts were amplified by PCR using primers 5'-AGCCCTCACTCCTTCTCTAG-3' and 5'-ACCTACAGGTGGGGTCTTTCATTCCC-3' and sequenced.

Generation and maintenance of cells expressing iRhom isoforms: Constructs of iRhom1-WT & iRhom1-ΔN, iRhom 2-WT & iRhom2-ΔN were constructed using three-step ligation. The 5' and 3' portions were amplified and restricted middle portion was ligated into modified version of

MSCVpuro (Clontech) containing a C-terminal T7 tag vector. Retrovirus encoding iRhom1-WT and iRhom1-ΔN, iRhom2-WT and iRhom2-ΔN, and empty pMSCVpuro alone (BD Biosciences), were produced in Phoenix ecotropic packaging cells and used to infect L-929 cells. Stable L-929 cells were generated through selection using puromycin (10 μg/ml). Unless otherwise indicated, cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 2μm of L-glutamine, 0.1 units of penicillin, 0.1μg/ml of streptomycin, antibiotics and 10% FCS.

Mouse embryonic fibroblasts were isolated from E13.5 wild type, ADAM17$^{-/-}$ (*4*), iR2$^{-/-}$ iR1$^{-/-}$ and iR1/2$^{-/-}$ (*10, 12, 15*) embryos to generate primary mEFs for immortalization. Briefly, head and viscera were removed and the remaining tissue were subjected to trypsin treatment for 15 min at 37 °C. Cells were collected and immortalized by transducing the plasmid expressing simian virus 40 (SV40) large T antigen maintained in (DMEM) supplemented with antibiotics and 10% FCS.

Immunofluorescence: Intracellular immunofluorescence staining was performed in L-929 cells stably expressing T7-iRhom2, T7-iRhom2-ΔN, T7-iRhom1, T7-iRhom1-ΔN and control vector, grown on glass cover slips, and fixed with 4% Formalin and where permeabilized with 1% Triton X-100. All iRhom versions were detected using a T7-Tag rabbit primary monoclonal antibody (mAb) at 1:300 (abcam) and donkey anti-rabbit Cy3-labelled secondary Ab (Jackson Immunolabs). The Golgi marker GM130 (BD eBiosciences) was recognized with a donkey anti-mouse Cy2-labelled secondary Ab (Jackson Immunolabs). Surface immunofluorescence staining of T7-iRhom2 and T7-iRhom2-ΔN cells was performed after fixation with 4% formalin. iRhom versions were detected using a T7-Tag rabbit monoclonal antibody (mAb) at 1:300 (abcam) and

donkey anti-rabbit Cy3-labelled secondary Ab (Jackson Immunolabs). Slides were stained with phalloidin FITC (Sigma) and hoechst 34580 (Invitrogen). Images were captured using confocal microscopy (Zeiss ELYRA).

Stable knockdown of ADAM17: Lentiviral particles were generated by calcium phosphate transfection of sub-confluent (50-60%) 293TV cells with 10 µg of shRNA (origene), 5 µg each of pMDG1.vsvg, pRSV-Rev and pMDLg/pRRE constructs. Lentiviral particles were collected 24 and 48 hours later, filtered through a 0.45 µm filter and stored at -80°C. Parental L929 cells were infected with lentiviral particles containing the indicated shRNAs and cells were selected with puromycin selection (48 hours, 5 µg/ml of puromycin).

ELISA detection of TNF and TNFR shedding: To analyze the TNFR shedding in L-929 cells overexpressing T7-iRhom2, T7-iRhom2-ΔN, T7-iRhom1, T7-iRhom1-ΔN and control vector were seeded (10^5) in 24-well plates. After 24 hours, supernatant was harvested and subjected to ELISA [DuoSet ELISA (R&D Systems)], which was performed according to the manufacturer's instructions. Cells were co-incubated with marimastat (BB 2516 20 µM, Tocris Bioscience) for 6 hours. For the treatment with GI or GW (Sigma) culture medium was removed and the cells were washed with PBS. Fresh FCS free DMEM containing 3µM of the inhibitors was added to the cells and the TNFR shedding was analyzed after 6 hours.

Similarly, for TNFR Shedding from TOC patient derived keratinocytes, $2x10^5$ cells per cell line were seeded into 24-well tissue culture plates. After 24 hours and 48 hours, supernatant was harvested and used for ELISA, performed according to the manufacturer's instructions (as above). To enable blockade of ADAM17, cells were co-incubated with ADAM17 inhibitor

TMI-005 (500 nM) or vehicle (DMSO). This medium was then collected after either 24 hours or 48 hours, and the concentration of TNFR therein was measured by ELISA. All experiments were carried out in triplicate.

Ectodomain shedding assay for AP-tagged KitL2: iRhom2 deficient fibroblasts were transfected with iRhom and AP-tagged KitL2 constructs using lipofectamine 2000 as previously described (*47*). 24 hours after transfection cells were incubated with marimastat (a kind gift from O. Ouerfelli, Sloan-Kettering Institute, New York, NY) or DPC-333/BMS-561392 (DPC; a kind gift from R. Waltermire, Brystol-Myers Squibb, New Brunswick, NJ) overnight or as indicated followed by three brief washes. Constitutive shedding was measured after 2 hours of subsequent incubation. Quantification of AP activity culture supernatant and cell lysate were performed by colorimetric assays as previously described (*47*).

Viability assay: L-929 cells overexpressing T7-iRhom2, T7-iRhom2-ΔN, T7-iRhom1, T7-iRhom1-ΔN and control vector were seeded (10^5) in 24-well plates. Same conditions were used for ADAM17 shRNA treated cells. Cells were incubated with recombinant mouse TNF (R&D Systems), with or without 20 μM BB 2516 (Tocris Bioscience). Viability was determined at indicated time points using Annexin V -7AAD exclusion (eBioscience) by flow cytometry(*48*).

Clonogenic assay: 10^5 L929 cells overexpressing T7-iRhom2, T7-iRhom2-ΔN, T7-iRhom1, T7-iRhom1-ΔN and control vector were seeded in 24-well plates. Cells were treated with recombinant mouse TNF (R&D Systems) at the indicated concentrations. After 48 hours cells were washed and stained with crystal violet at RT for 1 hour. The remaining crystal violet was

dissolved with methanol following analysis.

Reverse transcription PCR: RNA was extracted using RNeasy kit (Qiagen). mRNA expression of iRhom1-WT & iRhom1-ΔN, iRhom2-WT & iRhom2-ΔN was performed by reverse transcription PCR (Bio rad). For the analysis, the expression of the entire target mRNA was normalized to beta actin or GAPDH expression. Gene expression values were then calculated on the ΔΔCt method. Relative quantities (RQ) were determined with the equation: RQ = $2^{\wedge-\Delta\Delta Ct}$.

Cell surface protein isolation. Surface proteins from L-929 cells overexpressing T7-iRhom2, T7-iRhom2-ΔN or control vector were isolated using the sulfo-NHS-SS-biotin-based Cell Surface Protein Isolation Kit (Pierce) according to the manufacturers' instructions, after addition of 20 μM BB‐2516 (Tocris Bioscience) and 1,10-phenanthroline (Sigma) to the lysis and wash buffers. Cell surface fractions were compared to column flow-through (intracellular fractions) by immunoblotting.

Flow cytometry. Single cell suspensions from cultured cells were stained for 20 min at 4°C with T7 antibody in PBS containing 1% FCS and 5mM EDTA. Staining for Annexin V and 7AAD (eBioscience) was performed in solution containing 5 mM $Ca2^{+}$. Staining with TNFR1-biotin and TNFR2-biotin antibodies (eBioscience) was performed in PBS containing 1% FCS and 5mM EDTA for 1 hour at room temperature, followed by wash and incubation with PE-Cy7 coupled streptavidin labelled antibody (eBioscience) for 30min at 4°C.

Immunoprecipitation & immunoblotting: Briefly, cells were lysed in PBS containing 1% TX-100 (Sigma), EDTA-free protease inhibitor cocktail (Roche), Phospho stop (1 tablet/10mL), and the inhibitors BB-2516 (20μM; Tocris Bioscience) and 1,10-phenanthroline (10 mM; Sigma). The pulldown was performed using mouse mAbs recognizing the T7 tag or ADAM17 antibody (Abcam). To analyze the PARP-cleavage the cells were exposed to 2.5ng recombinant mouse TNF (R&D Systems) for indicated time points. Phospho-p65 or phospho-IκB-α activation was determined by harvesting cells from 60 mm dishes following treatment with 2.5 ng recombinant mouse TNF for indicated time points. After lysis the samples were used for immunoblotting using phospho-p65 and phospho-IκB-α antibodies (Cell Signaling).

Stability assay: 10^5 L-929 cells overexpressing T7-iRhom2, T7-iRhom2-ΔN or control vector were seeded in 24-well plates. Cells were incubated with 100 μg/mL cycloheximide (Sigma) for the indicated time points. After fixing cells using 4% formalin (Sigma) in PBS at room temperature for 40 min, cells were washed two times with and resuspended in PBST for 15 min at room temperature in the dark. After additional wash and suspension in PBS containing 1% FCS and 5mM EDTA, cells were stained with a T7 antibody (eBioscience) on ice for 30 min followed by measurement using flow cytometry.

Statistical analysis: Data are expressed as mean ± S.E.M or in S.D. Statistical significance between two groups was analyzed using the Mann–Whitney U test. For experiments involving analysis of multiple time points, two-way ANOVA with an additional Bonferroni post-test was used. *P* values < 0.05 were considered statistically significant. In all the experiments, n indicates the number of independent experiments performed.

References and Notes:

1. C. P. Blobel, ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* **6**, 32-43 (2005).
2. M. L. Moss, S. L. Jin, M. E. Milla, D. M. Bickett, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R. Didsbury, D. Hassler, C. R. Hoffman, T. A. Kost, M. H. Lambert, M. A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L. K. Overton, F. Schoenen, T. Seaton, J. L. Su, J. D. Becherer, et al., Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* **385**, 733-736 (1997).
3. R. A. Black, C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, D. P. Cerretti, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**, 729-733 (1997).
4. K. Horiuchi, T. Kimura, T. Miyamoto, H. Takaishi, Y. Okada, Y. Toyama, C. P. Blobel, Cutting edge: TNF-alpha-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. *Journal of immunology* **179**, 2686-2689 (2007).
5. J. J. Peschon, J. L. Slack, P. Reddy, K. L. Stocking, S. W. Sunnarborg, D. C. Lee, W. E. Russell, B. J. Castner, R. S. Johnson, J. N. Fitzner, R. W. Boyce, N. Nelson, C. J. Kozlosky, M. F. Wolfson, C. T. Rauch, D. P. Cerretti, R. J. Paxton, C. J. March, R. A. Black, An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281-1284 (1998).
6. P. Reddy, J. L. Slack, R. Davis, D. P. Cerretti, C. J. Kozlosky, R. A. Blanton, D. Shows, J. J. Peschon, R. A. Black, Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. *J Biol Chem* **275**, 14608-14614 (2000).
7. K. Pfeffer, T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Kronke, T. W. Mak, Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* **73**, 457-467 (1993).
8. J. Rothe, W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, H. Bluethmann, Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature* **364**, 798-802 (1993).
9. O. Micheau, J. Tschopp, Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* **114**, 181-190 (2003).
10. T. Maretzky, D. R. McIlwain, P. D. Issuree, X. Li, J. Malapeira, S. Amin, P. A. Lang, T. W. Mak, C. P. Blobel, iRhom2 controls the substrate selectivity of stimulated ADAM17-dependent ectodomain shedding. *Proc Natl Acad Sci U S A* **110**, 11433-11438 (2013).

11. C. Adrain, M. Zettl, Y. Christova, N. Taylor, M. Freeman, Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. *Science* **335**, 225-228 (2012).
12. D. R. McIlwain, P. A. Lang, T. Maretzky, K. Hamada, K. Ohishi, S. K. Maney, T. Berger, A. Murthy, G. Duncan, H. C. Xu, K. S. Lang, D. Haussinger, A. Wakeham, A. Itie-Youten, R. Khokha, P. S. Ohashi, C. P. Blobel, T. W. Mak, iRhom2 regulation of TACE controls TNF-mediated protection against Listeria and responses to LPS. *Science* **335**, 229-232 (2012).
13. O. M. Siggs, N. Xiao, Y. Wang, H. Shi, W. Tomisato, X. Li, Y. Xia, B. Beutler, iRhom2 is required for the secretion of mouse TNFalpha. *Blood* **119**, 5769-5771 (2012).
14. P. D. Issuree, T. Maretzky, D. R. McIlwain, S. Monette, X. Qing, P. A. Lang, S. L. Swendeman, K. H. Park-Min, N. Binder, G. D. Kalliolias, A. Yarilina, K. Horiuchi, L. B. Ivashkiv, T. W. Mak, J. E. Salmon, C. P. Blobel, iRHOM2 is a critical pathogenic mediator of inflammatory arthritis. *J Clin Invest* **123**, 928-932 (2013).
15. L. Xue, T. Maretzky, G. Weskamp, S. Monette, Qing X, P. Issureea, H. Crawford, D. McIlwain, T. Mak, J. Salmon, C. Blobel, iRhoms 1 and 2 are essential upstream regulators of ADAM17-dependent EGFR signaling. *Proc Natl Acad Sci U S A* **(In Press)**, (2015).
16. D. C. Blaydon, S. L. Etheridge, J. M. Risk, H. C. Hennies, L. J. Gay, R. Carroll, V. Plagnol, F. E. McRonald, H. P. Stevens, N. K. Spurr, D. T. Bishop, A. Ellis, J. Jankowski, J. K. Field, I. M. Leigh, A. P. South, D. P. Kelsell, RHBDF2 mutations are associated with tylosis, a familial esophageal cancer syndrome. *American journal of human genetics* **90**, 340-346 (2012).
17. S. Saarinen, P. Vahteristo, R. Lehtonen, K. Aittomaki, V. Launonen, T. Kiviluoto, L. A. Aaltonen, Analysis of a Finnish family confirms RHBDF2 mutations as the underlying factor in tylosis with esophageal cancer. *Familial cancer* **11**, 525-528 (2012).
18. M. A. Brooke, S. L. Etheridge, N. Kaplan, C. Simpson, E. A. O'Toole, A. Ishida-Yamamoto, O. Marches, S. Getsios, D. P. Kelsell, iRHOM2-dependent regulation of ADAM17 in cutaneous disease and epidermal barrier function. *Human molecular genetics* **23**, 4064-4076 (2014).
19. S. L. Etheridge, M. A. Brooke, D. P. Kelsell, D. C. Blaydon, Rhomboid proteins: a role in keratinocyte proliferation and cancer. *Cell and tissue research* **351**, 301-307 (2013).
20. O. M. Siggs, A. Grieve, H. Xu, P. Bambrough, Y. Christova, M. Freeman, Genetic interaction implicates iRhom2 in the regulation of EGF receptor signalling in mice. *Biol Open* **3**, 1151-1157 (2014).
21. V. Hosur, K. R. Johnson, L. M. Burzenski, T. M. Stearns, R. S. Maser, L. D. Shultz, Rhbdf2 mutations increase its protein stability and drive EGFR hyperactivation through enhanced secretion of amphiregulin. *Proc Natl Acad Sci U S A* **111**, E2200-2209 (2014).
22. D. Vercammen, R. Beyaert, G. Denecker, V. Goossens, G. Van Loo, W. Declercq, J. Grooten, W. Fiers, P. Vandenabeele, Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med* **187**, 1477-1485 (1998).

23. M. Tsujimoto, Y. K. Yip, J. Vilcek, Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc Natl Acad Sci U S A* **82**, 7626-7630 (1985).
24. N. Vanlangenakker, M. J. Bertrand, P. Bogaert, P. Vandenabeele, T. Vanden Berghe, TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. *Cell Death Dis* **2**, e230 (2011).
25. D. Bhattacharya, E. C. Logue, S. Bakkour, J. DeGregori, W. C. Sha, Identification of gene function by cyclical packaging rescue of retroviral cDNA libraries. *Proc Natl Acad Sci U S A* **99**, 8838-8843 (2002).
26. M. Irmler, M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J. L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L. E. French, J. Tschopp, Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190-195 (1997).
27. B. Liu, Y. Xu, W. L. Li, L. Zeng, Proteomic analysis of differentially expressed skin proteins in iRhom2(Uncv) mice. *BMB Rep* **48**, 19-24 (2015).
28. C. Adrain, M. Freeman, New lives for old: evolution of pseudoenzyme function illustrated by iRhoms. *Nat Rev Mol Cell Biol* **13**, 489-498 (2012).
29. K. Azijli, B. Weyhenmeyer, G. J. Peters, S. de Jong, F. A. Kruyt, Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ* **20**, 858-868 (2013).
30. C. J. Kearney, S. P. Cullen, G. A. Tynan, C. M. Henry, D. Clancy, E. C. Lavelle, S. J. Martin, Necroptosis suppresses inflammation via termination of TNF- or LPS-induced cytokine and chemokine production. *Cell Death Differ*, (2015).
31. D. R. McIlwain, T. Berger, T. W. Mak, Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* **5**, a008656 (2013).
32. A. Strelow, K. Bernardo, S. Adam-Klages, T. Linke, K. Sandhoff, M. Kronke, D. Adam, Overexpression of acid ceramidase protects from tumor necrosis factor-induced cell death. *J Exp Med* **192**, 601-612 (2000).
33. D. F. Legler, O. Micheau, M. A. Doucey, J. Tschopp, C. Bron, Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. *Immunity* **18**, 655-664 (2003).
34. C. Hundhausen, D. Misztela, T. A. Berkhout, N. Broadway, P. Saftig, K. Reiss, D. Hartmann, F. Fahrenholz, R. Postina, V. Matthews, K. J. Kallen, S. Rose-John, A. Ludwig, The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood* **102**, 1186-1195 (2003).
35. Y. Christova, C. Adrain, P. Bambrough, A. Ibrahim, M. Freeman, Mammalian iRhoms have distinct physiological functions including an essential role in TACE regulation. *EMBO Rep* **14**, 884-890 (2013).
36. M. Zettl, C. Adrain, K. Strisovsky, V. Lastun, M. Freeman, Rhomboid family pseudoproteases use the ER quality control machinery to regulate intercellular signaling. *Cell* **145**, 79-91 (2011).
37. S. M. Le Gall, T. Maretzky, P. D. Issuree, X. D. Niu, K. Reiss, P. Saftig, R. Khokha, D. Lundell, C. P. Blobel, ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site. *J Cell Sci* **123**, 3913-3922 (2010).

38. T. Maretzky, A. Evers, W. Zhou, S. L. Swendeman, P. M. Wong, S. Rafii, K. Reiss, C. P. Blobel, Migration of growth factor-stimulated epithelial and endothelial cells depends on EGFR transactivation by ADAM17. *Nat Commun* **2**, 229 (2011).
39. D. Nitoiu, S. L. Etheridge, D. P. Kelsell, Insights into desmosome biology from inherited human skin disease and cardiocutaneous syndromes. *Cell Commun Adhes* **21**, 129-140 (2014).
40. F. R. Hirsch, M. Varella-Garcia, F. Cappuzzo, Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. *Oncogene* **28 Suppl 1**, S32-37 (2009).
41. S. Mochizuki, Y. Okada, ADAMs in cancer cell proliferation and progression. *Cancer Sci* **98**, 621-628 (2007).
42. Y. Leilei, L. Bing, L. Yang, W. Shaoxia, X. Yuan, W. Dongping, Y. Huahu, S. Shichen, Z. Guangzhou, P. Ruiyun, Z. Lin, L. Wenlong, iRhom2 mutation leads to aberrant hair follicle differentiation in mice. *PLoS One* **9**, e115114 (2014).
43. J. R. Doedens, R. A. Black, Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme. *J Biol Chem* **275**, 14598-14607 (2000).
44. T. Nakagawa, A. Guichard, C. P. Castro, Y. Xiao, M. Rizen, H. Z. Zhang, D. Hu, A. Bang, J. Helms, E. Bier, R. Derynck, Characterization of a human rhomboid homolog, p100hRho/RHBDF1, which interacts with TGF-alpha family ligands. *Dev Dyn* **233**, 1315-1331 (2005).
45. H. Zou, S. M. Thomas, Z. W. Yan, J. R. Grandis, A. Vogt, L. Y. Li, Human rhomboid family-1 gene RHBDF1 participates in GPCR-mediated transactivation of EGFR growth signals in head and neck squamous cancer cells. *FASEB J* **23**, 425-432 (2009).
46. Z. Yan, H. Zou, F. Tian, J. R. Grandis, A. J. Mixson, P. Y. Lu, L. Y. Li, Human rhomboid family-1 gene silencing causes apoptosis or autophagy to epithelial cancer cells and inhibits xenograft tumor growth. *Mol Cancer Ther* **7**, 1355-1364 (2008).
47. T. Maretzky, W. Zhou, X. Y. Huang, C. P. Blobel, A transforming Src mutant increases the bioavailability of EGFR ligands via stimulation of the cell-surface metalloproteinase ADAM17. *Oncogene* **30**, 611-618 (2011).
48. M. Grusdat, D. R. McIlwain, H. C. Xu, V. I. Pozdeev, J. Knievel, S. Q. Crome, C. Robert-Tissot, R. J. Dress, A. A. Pandyra, D. E. Speiser, E. Lang, S. K. Maney, A. R. Elford, S. R. Hamilton, S. Scheu, K. Pfeffer, J. Bode, H. W. Mittrucker, M. Lohoff, M. Huber, D. Haussinger, P. S. Ohashi, T. W. Mak, K. S. Lang, P. A. Lang, IRF4 and BATF are critical for CD8(+) T-cell function following infection with LCMV. *Cell Death Differ* **21**, 1050-1060 (2014).

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Figure Legends:

Fig 1: N-truncated iRhom1-ΔN confers TNF resistance as identified by CPR screening.

(A) PCR results from each round in the CPR screen (see Methods) showing enrichment for cFLIP and two short versions of iRhom1 in TNF-resistant cells. **(B)** Systematic representation of wild-type (WT) and short versions of iRhom1 identified by CPR relative to their predicted transmembrane domain structures. **(C)** Immunoblotting and densitometry for T7 in lysates from

L-929 cells expressing a control vector, or T7-tagged wild-type (WT) or ΔN iRhom1 (iR1) or iRhom2 (iR2) (n=3). **(D and E)** Cell death, assessed by Annexin V binding and 7-AAD staining using flow cytometry, in 10^5 L-929 cells transfected as indicated and treated with recombinant TNF for up to 48 hours. ($n \geq 5$). Data are mean ± S.E.M. from the number of experiments (n) indicated; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ against ΔN; # $p<0.05$, ## $p<0.01$ against vector.

Fig 2: iRhom2-ΔN induces TNFR shedding.

(A and B) Immunoblotting and densitometry for PARP (A) or total and phosphorylated NF-κB pathway proteins (B) in lysates from L-929 cells overexpressing a vector control, iRhom2-WT or iRhom2-ΔN and exposed to recombinant TNF (2.5 ng/ml) for up to 8 hours (A) or 45 min (B). (n=3, normalized to control and actin). **(C and D)** Flow cytometry analysis of TNFR1 and TNFR2 surface abundance on L929 cells overexpressing wild-type or truncated iRhom2 (C) or iRhom1 (D). (n=5 or 6, respectively). **(E and F)** Amount of TNFR1 and TNFR2 in supernatants from 10^5 L-929 cells expressing wild-type or truncated iRhom2 (E) or iRhom1 (F). (n=5). Data are mean ± S.E.M. from the number of experiments (n) indicated; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ against ΔN; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ against vector.

Fig 3: Effects of ΔN iRhoms can be blocked by ADAM17 inhibitors.

(A and B) Abundance of TNFR1 and TNFR2 in the culture supernatants from 10^5 L-929 cells expressing wild-type or truncated iRhom2 cultured in presence or absence of (A) marimastat

(MM, 20 μM) or (B) GI or GW (each 3 μM) for 6 hours. (n=5 or 6, respectively). **(C)** As in (B) in10^5 L-929 cells expressing wild-type or truncated iRhom1. **(D)** Cell death as a percentage of Annexin7^+-AAD^+ cells in cultures (n=3) of 10^5 L-929 cells expressing wild-type or truncated iRhom2 treated with recombinant TNF in the presence or absence of marimastat (MM, 20 μM). Data are mean ± S.E.M. from the number of experiments (n) indicated; ** $p<0.01$, *** $p<0.001$ against wild-type; # $p<0.05$, ### $p<0.001$ against vector.

Fig 4: Stable knockdown of ADAM17 prevents increased TNFR shedding and resistance to TNF.

(A) Immunoblot for pro (P) and mature (M) ADAM17 in lysates from L-929 cells expressing full-length and ΔN iRhom2 transfected with either a scrambled control (Scr1) or an *ADAM17*-targeted shRNA (ADAM17-Sh1). Blot is representative of 3 experiments. **(B)** Surface abundance of TNFR1 and TNFR2 as determined by flow cytometry on L-929 cells expressing a vector control, full-length (WT) iRhom2 or iRhom2-ΔN in the presence of either control or ADAM17 shRNA (n=6). **(C and D)** Cell death as a proportion of Annexin7^+-AAD^+ cells in cultures of 10^5 L-929 cells stably expressing full-length or ΔN iRhom2 (C) or iRhom1 (D) and either scrambled or ADAM17 shRNA treated with recombinant TNF for 48 hours. (n=4 or 6, respectively). Data are mean ± S.E.M. from the number of experiments (n) indicated; * $p<0.05$, *** $p<0.001$.

Fig 5: Truncation of the cytoplasmatic tail results in increased surface expression of iRhom2.

(A) Immunoprecipitation (IP) for T7 or ADAM17 followed by immunoblotting for the same in lysates from L-929 cells stably expressing T7-tagged wild-type or truncated iRhom2. P, pro and M, mature ADAM17. Blot is representative of 3 experiments. **(B)** Immunoblotting for iRhom2 using a T7 antibody in intracellular and cell surface fractions from L-929 cells stably expressing wild-type or truncated iRhom2. Blot is representative of 3 experiments. **(C)** Surface abundance of iRhom2, determined using an antibody against T7, on stably transfected L-929 cells (n=8). **(D)** Immunocytochemistry for iRhom2 using T7-antibodies (Cy3), Phalloidin-FITC and Hoechst staining in stably transfected L-929 cells, fixed and/or permeabilized as indicated (n≥3 experiments). **(E)** Flow cytometry analysis of mean fluorescence intensity (MFI) of the surface abundance of iRhom2 on unpermeabilized stably transfected L-929 cells expressing either scrambled or ADAM17 shRNA (E; n≥4 experiments) or immortalized wild-type or *Adam17* knockout MEFs (F; n=12 experiments). Data are mean ± S.E.M. from the number of experiments (n) indicated; *** $p<0.001$.

Fig 6: N-terminal mutations in iRhom2 trigger constitutive activity of ADAM17.

(A and B) TNFR1 and TNFR2 in conditioned media 10^5 L-929 cells expressing wild-type or ΔN iRhom2 (A) or iRhom1 (B) cultured with PMA (100 ng/ml) in the presence (where indicated) of GI or GW (each 3 μM) (n=6). **(C)** KitL2 shedding in immortalized MEFs genetically lacking iRhom2 transfected with AP-tagged KitL2 along with MAD2 (control), iRhom2WT, or iRhom2-ΔN, and (where indicated, +) preincubated with marimastat overnight (MM/ON), followed by washout of the inhibitor (n=3). **(D)** TGF-α shedding from immortalized

MEFs genetically lacking iRhom1 and iRhom2 and transfected with AP-tagged TGF-α along with MAD2 (control), murine wild-type iRhom2 or iRhom2$^{I156T + P159L}$ (n≥5). **(E)** Concentration of soluble TNFR1 in supernatants from 2 x 10^5 keratinocytes from either healthy donors (K17 cells) or tylosis patients that have mutations in IRHOM2 $^{(I186T/WT)}$ [TYLK1 and TYLK2 treated with vehicle (DMSO) or TMI-005 ADAM17 inhibitor for 24 (left) or 48 (right) hours. Data are mean ± S.E.M. from the number of experiments (n) indicated; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ against wild-type (A and B) or MAD2 (D and E); # $p<0.05$ against vector (A and B) or mutant construct (D and E).