ADAM17 stabilizes its interacting partner inactive Rhomboid 2 (iRhom2) but not inactive Rhomboid 1 (iRhom1)

The metalloprotease ADAM17 (a disintegrin and metalloprotease 17) is a key regulator of tumor necrosis factor α (TNFα), interleukin 6 receptor (IL-6R), and epidermal growth factor receptor (EGFR) signaling. ADAM17 maturation and function depend on the seven-membrane-spanning inactive rhomboid-like proteins 1 and 2 (iRhom1/2 or Rhbdf1/2). Most studies to date have focused on overexpressed iRhom1 and -2, so only little is known about the properties of the endogenous proteins. Here, we show that endogenous iRhom1 and -2 can be cell surface–biotinylated on mouse embryonic fibroblasts (mEFs), revealing that endogenous iRhom1 and -2 proteins are present on the cell surface and that iRhom2 also is present on the surface of lipopolysaccharide–stimulated primary bone marrow–derived macrophages. Interestingly, very little, if any, iRhom2 was detectable in mEFs or bone marrow–derived macrophages lacking ADAM17, suggesting that iRhom2 is stabilized by ADAM17. By contrast, the levels of iRhom1 were slightly increased in the absence of ADAM17 in mEFs, indicating that its stability does not depend on ADAM17. These findings support a model in which iRhom2 and ADAM17 are obligate binding partners and indicate that iRhom2 stability requires the presence of ADAM17, whereas iRhom1 is stable in the absence of ADAM17.

A disintegrin and metalloprotease 17 (ADAM17) is a cell-surface metalloprotease that is required for the proteolytic processing of tumor necrosis factor α (TNFα) and is therefore also referred to as TACE (TNFα convertase). In addition, ADAM17 has a crucial role in the proteolytic release and activation of several ligands of the epidermal growth factor receptor (EGFR) as well as of the IL-6 receptor (IL-6R) and other membrane proteins (1–7). Major functions of ADAM17 include the regulation of the EGFR signaling pathway during development (4–8) and protection of the skin and intestinal barrier in adults (9–10). Moreover, ADAM17 can contribute to cancers that involve inappropriate EGFR signaling (15, 16) and to pathologies involving dysregulated TNFα and IL-6R pathways, including autoimmune diseases such as rheumatoid arthritis (17, 18).

ADAM17 can be rapidly and post-translationally activated by a number of different signaling pathways (19–23) and requires its transmembrane domain, but not its cytoplasmic domain, for this rapid posttranslational activation (19). The seven-membrane–spanning protein iRhom2 (inactive Rhomboid 2, also referred to as Rhbdf2, Rhomboid 5 homolog 2) was identified as a crucial regulator of the maturation of ADAM17 in bone marrow–derived macrophages (BMDM) (24, 25). Additional insight into the relationship of ADAM17 and iRhom2 was provided by a point mutation in the first transmembrane domain (TMD) of iRhom2, termed sinecure, which results in a

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2The abbreviations used are: ADAM17, a disintegrin and metalloprotease 17; Rhbdf1/2, Rhomboid 5 homolog 1/2; iRhom1/2, inactive Rhomboid-like protein 1/2; TNFα, tumor necrosis factor α; ER, endoplasmic reticulum; IL-6, interleukin 6; IL-6R, interleukin-6 receptor; TMD, transmembrane domain; LPS, lipopolysaccharide; BMDM, bone marrow–derived macrophage(s); EndoH, endoglycosidase H; PNGase F, N-glycosidase F; EGFR, epidermal growth factor receptor; STING, stimulator of interferon genes; NLDL, newborn liver–derived macrophage(s); pAb, polyclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P, postnatal day; E, embryonic day; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; qPCR, quantitative PCR; HRP, horseradish peroxidase.
strong reduction of ADAM17-dependent TNFα release from BMDM (26). Mice that are homozygous for the iRhom2 sine-
cure mutation and also lack the related iRhom1 resemble previ-
ously described iRhom1/2−/− double knockout mice (27, 28),
demonstrating that the sinecure point mutation in the first
TMD of iRhom2 results in a strongly hypomorphic phenotype.
Moreover, the substrate selectivity of ADAM17 is differentially
regulated by iRhom1 and -2 (29), and point mutations in the
TMD of ADAM17 that were predicted to affect the interaction
with iRhom2 strongly reduced iRhom2/ADAM17-dependent
shedding events, without affecting iRhom1/ADAM17-dependent
shedding (28). The different substrate selectivity of
iRhom2/ADAM17 and iRhom1/ADAM17-dependent shed-
ing (29), the effects of the sinecure mutation on ADAM17, and
the effects of point mutations in the TMD of ADAM17 on
ADAM17/iRhom2-dependent shedding (28) suggested that
iRhom2 and ADAM17 form a heteromeric complex. Presum-
ably, this complex associates in the endoplasmic reticulum (ER)
and remains together to regulate iRhom2/ADAM17-depen-
dent shedding on the cell surface or in the late secretory path-
way. This model is further supported by recent studies demon-
strating that mutations in cytoplasmic phosphorylation sites of
iRhom2 affect the activation of ADAM17 (30, 31). In addition, a
newly discovered iRhom2-interacting protein, termed FrmΔ8
(FERM domain—containing 8) or iTAP (iRhom tail–associated
protein), was found to regulate endocytosis of iRhom2/
ADAM17 and degradation in the lysosome (32, 33).

Most studies on iRhom1 and -2 to date have focused on the
overexpressed proteins (29–34), so there is a paucity of infor-
mation on the cell biological properties of endogenous iRhom1
and only limited information on iRhom2 (25). The main goal of
the current study was to perform a biochemical characteriza-
tion of endogenous iRhom2 using primary mouse macrophage
cultures and mouse embryonic fibroblasts. In light of the cru-
icial role of iRhom2 in regulating the maturation and function of
ADAM17 (27), we were also interested in whether ADAM17
reciprocally affects the stability of iRhom2 and the related
iRhom1 and their transport to the cell surface. Moreover,
because iRhom2 has been shown to interact with the multi-
membrane-spanning protein stimulator of interferon genes
(STING) (35), this raised questions about the role of STING in
the stability and maturation of iRhom2 and ADAM17.

Results

Characterization of murine iRhom2 in primary bone marrow–
derived macrophages

To characterize endogenous mouse iRhom2, we raised rabbit
polycyal antibodies (pAbs) against a portion of the N-termi-
nal cytoplasmic domain of murine iRhom2 (amino acid resi-
dues 1–376; see “Experimental procedures” for details). These
anti-iRhom2 pAbs were tested on lysates of primary BMDM
isolated from iRhom2−/− mice or WT controls. Because the expres-
sion of iRhom2 is up-regulated by treatment with LPS
(36), we compared untreated BMDM with cells that had been
stimulated with 10 ng/ml LPS overnight. LPS treatment
induced a band of ~95 kDa in WT BMDM (marked by an aster-
isk in Fig. 1A (top)) that was not present in iRhom2−/− BMDM.

Figure 1. Western blot analysis of mouse iRhom2 in cell lysates and puri-
ified membranes isolated from mouse BMDM. A, primary BMDM isolated
from WT or iRhom2−/− mice were either not treated or incubated with 10
ng/ml LPS for 14–18 h and then lysed and subjected to Western blot analysis.
B, Western blotting of high-speed membrane preparations from LPS-treated
WT or iRhom2−/− BMDM showed a strong enrichment of iRhom2 and much
weaker nonspecific bands compared with the whole-cell lysate. The samples
were either probed with rabbit polyclonal antibodies against the cytoplasmic
domain of mouse iRhom2 (top panels), or antibodies against the cytoplasmic
domain of ADAM17 (middle panels), or tubulin or ADAM10, as indicated (bot-
tom panels). The heavy line in A (middle) indicates splicing of nonadjacent
lanes. Each panel is representative of three separate experiments.

However, the anti-iRhom2 pAbs also reacted with several other
proteins on blots of the BMDM lysates that were present in
iRhom2−/− deficient samples, and thus nonspecific (Fig. 1A, top).
The LPS treatment strongly induced the levels of pro- and
mature ADAM17 in WT BMDM but only of pro-ADAM17 in
iRhom2−/− BMDM (Fig. 1A (middle); tubulin served as loading
control (bottom)).

To improve detection of the seven-membrane–spanning
iRhom2 and remove nonspecifically recognized soluble pro-
teins, we purified cellular membranes by high-speed centri-
figuration to enrich for membrane proteins (see “Experimental
procedures” for details). Western blot analysis of the purified
material using the same anti-iRhom2(1–376) rabbit pAbs
revealed a strong band of ~95 kDa in the LPS-treated WT
BMDM sample that was not present in the identically prepared
iRhom2−/− control (Fig. 1B, top). Moreover, both pro- and
mature ADAM17 were present in the whole lysate and mem-
brane preparations from WT BMDM, whereas only pro-
ADAM17 could be detected in the iRhom2−/− sample, but not
mature ADAM17, as reported previously (24, 25) (Fig. 1, A and
B, middle panels); the membrane-anchored ADAM10 (A10)
served as a loading control in the bottom panel of Fig. 1B).

Cell-surface biotinylation of endogenous iRhom2 in
LPS-stimulated BMDM

To establish whether the endogenous iRhom2 is present on
the cell surface of LPS-treated BMDM, a non-membrane-per-
measurable biotinylation reagent (Sulfo-NHS-LC-Biotin) was used to label cell-surface proteins on these cells (see “Experimental procedures” for details). When a Western blot of the purified biotinylated material was probed with anti-iRhom2 pAbs, a band of 95 kDa was detected in WT BMDM, but not in iRhom2−/− BMDM (Fig. 2, top). Endoglycosidase H (EndoH) typically cannot process N-linked carbohydrates from glycoproteins that have migrated through the medial Golgi apparatus. However, EndoH treatment of purified cell-surface iRhom2 led to slightly faster migration of cell surface–biotinylated iRhom2 (Fig. 2, top). When we instead treated these same samples with protein-N-glycanase F (PNGase F), which removes all N-linked carbohydrate residues, iRhom2 migrated faster than the EndoH-treated sample (Fig. 2, top). The partial susceptibility of N-linked carbohydrates in cell surface–biotinylated iRhom2 to treatment with EndoH was reminiscent of the effect of EndoH and PNGase F in Western blots of total iRhom2 in BMDM lysates in a prior report (25). The finding that the surface–labeled iRhom2 is partially susceptible to EndoH treatment demonstrates that iRhom2 progresses through the medial Golgi apparatus on the way to the cell surface and that at least one N-linked carbohydrate residue in iRhom2 does not acquire EndoH resistance (25). Similarly, blots of the cell surface–biotinylated ADAM17 in WT BMDM showed resistance to EndoH (Fig. 2, left), but sensitivity to PNGase F, consistent with previous studies on mature ADAM17 (25, 37). These results also corroboration that no mature ADAM17 could be biotinylated on the surface of iRhom2−/− BMDM (24, 25). Finally, cell surface–biotinylated ADAM9 was included as a control for the iRhom2−/− BMDM. Like iRhom2, cell surface–labeled ADAM9 is also partially sensitive to EndoH treatment, as described previously (38). To rule out nonspecific binding of iRhom2, ADAM17, or ADAM9 to the streptavidin-Sepharose beads used to precipitate cell surface–biotinylated proteins, we incubated extracts of equivalent cultures of WT cells that were either untreated or cell surface–biotinylated with streptavidin beads and performed a Western blot analysis on the bound proteins (Fig. 2B). These experiments confirmed that only the biotinylated forms of iRhom2, ADAM17, and ADAM9 bound to streptavidin beads under the conditions used here, whereas these proteins in the lysate of an equivalent number of untreated cells did not. The finding that endogenous iRhom2 can be biotinylated using a non-membrane-permeable biotinylation reagent demonstrates that endogenous iRhom2 is present on the cell surface of primary BMDM, similar to RAW264.7 cells (31).

**ADAM17 is required for the stabilization of iRhom2**

iRhom2-deficient BMDM lack mature ADAM17, whereas the levels of pro-ADAM17 do not appear to be significantly affected (24, 25). This raises questions about whether the loss of ADAM17 would reciprocally affect the levels of iRhom2. Because Adam17−/− mice die at birth, we isolated macrophages from the livers of newborn Adam17−/− mice and their WT control littersmates to assess the fate of iRhom2 in myeloid cells in the absence of ADAM17. iRhom2 was only weakly detectable in membrane preparations of unstimulated newborn liver-derived macrophages (NLDM) from WT mice (Fig. 3A, top, left lane). However, stimulation with 10 ng/ml LPS increased the production of both iRhom2 and ADAM17 in WT NLDM (Fig. 3A, top and middle panels, right lane), just as in BMDM from adult mice (Fig. 1). Interestingly, iRhom2 protein could not be detected by the polyclonal iRhom2 antibody in a Western blot analysis of membrane preparations of LPS-stimulated Adam17−/− NLDM (Fig. 3B, iRhom2 (top), ADAM17 (middle), and ADAM10 control (bottom)).

To further explore the role of ADAM17 in stabilizing iRhom2 in mouse embryos, isolated membrane preparations
from extracts of newborn mice (see “Experimental procedures” for details) were probed for iRhom2 or ADAM17. No iRhom2 could be detected in embryos lacking ADAM17 or iRhom2, although both ADAM17 and iRhom2 were present in WT control extracts (Fig. 3C). Moreover, pro- and mature ADAM17 were present in extracts of newborn iRhom2−/− mice, where maturation of ADAM17 is supported by iRhom1 (27).

**Analysis of mRNA expression**

The stabilization of iRhom2 by ADAM17 could depend on the requirement for a continuous interaction of the two heteromeric binding partners, or alternatively, ADAM17 may have a role in controlling the transcription of iRhom2. We therefore performed a qPCR analysis of the expression of iRhom2 mRNA in WT and ADAM17−/− BMDM (A17−/−), but not in iRhom2−/− BMDM (IR2−/−), used here as a control. B, LPS-stimulated BMDM from WT mice were treated with or without 5 µM marimastat for 18 h and then lysed, and the samples were subjected to Western blot analysis for iRhom2 or ADAM17, with ADAM10 as a loading control. The heavy line in the top panel (IR2 samples) indicates that these two lanes were spliced together. Each experiment was repeated three times with essentially similar outcomes, and one representative sample is shown.

**Effect of protein degradation inhibitors on iRhom2 stability**

We next considered possible degradation pathways for iRhom2 in the absence of ADAM17. We therefore incubated LPS-stimulated BMDM from mice, in which floxed alleles of ADAM17 were conditionally inactivated in myeloid cells through expression of LysM-Cre (A17LysM-Cre mice) with an inhibitor of proteasomal degradation (MG132, 10 µM) (30, 39), an inhibitor of ER-associated degradation (eeyarestatin, 10 µM) (40), and inhibitors of lysosomal acidification and autophago-

**iRhom2 is stabilized by ADAM17, but iRhom1 is not**

![Figure 4. Expression analysis of iRhom2 by RT-qPCR and effect of treatment of WT cells with the metalloprotease inhibitor marimastat. A, an RT-qPCR analysis shows comparable expression of iRhom2 mRNA in WT and ADAM17−/− BMDM (A17−/−), but not in iRhom2−/− BMDM (IR2−/−), used here as a control. B, LPS-stimulated BMDM from WT mice were treated with or without 5 µM marimastat for 18 h and then lysed, and the samples were subjected to Western blot analysis for iRhom2 or ADAM17, with ADAM10 as a loading control. The heavy line in the top panel (IR2 samples) indicates that these two lanes were spliced together. Each experiment was repeated three times with essentially similar outcomes, and one representative sample is shown.](image)

**Figure 5. Effect of inhibitors of endocytosis or protein degradation on iRhom2 levels in ADAM17-deficient cells.** BMDM from Adam17-LysM-cre mice (A) or Adam17−/− mEFs (B) were either left untreated or treated with 10 µM MG132, 100 µM chloroquine, or 10 µM eeyarestatin I, as indicated, for 18 h and then subjected to Western blot analysis for iRhom2, ADAM17, or ADAM10 and compared with untreated WT BMDM (A) or mEFs (B). As controls for the efficacy of the inhibitors, WT mEFs were incubated under identical conditions as the ADAM17-deficient BMDM (A) or mEFs (B). Whole-cell lysates of the WT mEFs treated with eeyarestatin I or MG132 were subjected to Western blot analysis with antibodies against ubiquitin (C), and Western blots of extracts from WT mEFs treated with bafilomycin or chloroquine were probed for LC3-II, a marker for inhibition of autophagy (D). The data are representative of three replicates with essentially similar results.

**STING is not required for the stability of iRhom2**

The multimembrane-spanning protein STING has been reported as an interacting partner of iRhom2 (35). To determine whether STING is required to stabilize iRhom2, we isolated BMDM from Sting−/− mice, or from WT controls. As shown in Fig. 6A, the lack of STING had no detectable effect on the protein levels of iRhom2 or ADAM17. Moreover, we found that inactivation of iRhom2 in BMDM also did not have a strong effect on the levels of STING that were detectable by Western blotting (Fig. 6B). Finally, the mRNA levels for iRhom2 were comparable in BMDM from WT and Sting−/− mice, but undetectable in BMDM from iRhom2−/− mice (Fig. 6C).
proteins, in this case pro-ADAM17 or pro-ADAM9.

Specificity of these mAbs. We noted that the levels of iRhom2 were not significantly affected by the absence of iRhom1 and that the anti-iRhom2 rat mAb recognized a small but detectable amount of iRhom2 in A17−/− mEFs. The rat mAb against iRhom1 demonstrated that the levels of iRhom1 were not significantly changed in iRhom2−/− mEFs, but they appeared slightly increased in the A17−/− mEFs. Cell-surface biotinylation of WT mEFs showed that both iRhom1 and iRhom2 could be detected on the cell surface of these cells (Fig. 7B). The cell surface biotinylation of only the mature form of ADAM17 and of ADAM9, used as loading control, but not their pro-forms served as an internal control that the biotinylation reagent was specific for cell-surface proteins and did not label intracellular proteins, in this case pro-ADAM17 or pro-ADAM9.

Discussion

Previous studies have shown that pro-ADAM17 is synthesized in the absence of iRhom2 in BMDM, but not transported out of the ER to the trans-Golgi network, where its pro-domain is removed (24, 45). This finding raised questions about the fate of iRhom2 in the absence of ADAM17 or in the absence of a recently identified iRhom2-binding partner, STING. Moreover, because mEFs lacking both iRhom1 and iRhom2 only have pro-ADAM17, but no detectable mature ADAM17, we were interested in exploring whether endogenous iRhom2 and the related iRhom1 can be detected on the cell surface of WT mEFs, as would be predicted if they can function as regulators of endogenous mature ADAM17. Finally, we were interested in whether the lack of ADAM17 reciprocally affects the stability of iRhom1 and -2.

Our observation that little, if any, iRhom2 is detectable by Western blotting in myeloid cells or in embryos lacking ADAM17 under conditions where it can readily be detected in WT controls provides the first evidence that ADAM17 is required for the stabilization of endogenous iRhom2. Unlike pro-ADAM17, which is present at comparable levels in WT and iRhom2−/− BMDM and can be up-regulated by treatment of BMDM with LPS, we found no detectable iRhom2 in membrane preparations of LPS-treated Adam17−/− NLDM. Treatment with inhibitors of proteasomal degradation, ER-associated degradation, or lysosomal acidification and degradation did not restore the ability to detect iRhom2 in Adam17−/− NLDM or in Adam17−/− mEFs, arguing against a major role of these pathways in controlling the stability of iRhom2 in the

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Figure 6. Expression of STING, iRhom2, and ADAM17 in BMDM lacking STING or iRhom2. Western blot analysis for iRhom2, ADAM17, Sting, or ADAM10 was performed on unstimulated or LPS-stimulated BMDM from Sting−/− mice or wild type (WT) controls (A) or from iRhom2−/− mice and WT controls (B). The heavy line in B indicates splicing of nonadjacent lanes. An RT-qPCR analysis was used to confirm that the mRNA for iRhom2 was expressed at similar levels in Sting−/− BMDM compared with WT controls. Each experiment was repeated three times, and representative samples are shown. Error bars, S.E.M.

Figure 7. Rat monoclonal antibodies against iRhom1 or -2 allow detection of the endogenous proteins in mEFs lacking ADAM17

When we attempted to detect the endogenous iRhom2 in Western blots of mouse embryonic fibroblasts, we found that the rabbit anti-iRhom2(1–376) polyclonal antibodies against the cytoplasmic domain of iRhom2 were not reproducibly effective for this purpose (data not shown). Therefore, new rat mAbs against mouse iRhom1 or iRhom2 were generated (see “Experimental procedures” for details). As shown in Fig. 7A, the rat mAbs specifically recognized iRhom1 or -2 in WT mEFs, with mEFs lacking iRhom1 (iR1KO) or iRhom2 (iR2KO) or both iRhom1 and -2 (iR1/2DKO) serving as controls for the specificity of these mAbs. We noted that the levels of iRhom2 were not significantly affected by the absence of iRhom1 and that the anti-iRhom2 rat mAb recognized a small but detectable amount of iRhom2 in A17−/− mEFs. The rat mAb against iRhom1 demonstrated that the levels of iRhom1 were not significantly changed in iRhom2−/− mEFs, but they appeared slightly increased in the A17−/− mEFs. Cell-surface biotinylation of WT mEFs showed that both iRhom1 and iRhom2 could be detected on the cell surface of these cells (Fig. 7B). The cell surface biotinylation of only the mature form of ADAM17 and of ADAM9, used as loading control, but not their pro-forms served as an internal control that the biotinylation reagent was specific for cell-surface proteins and did not label intracellular proteins, in this case pro-ADAM17 or pro-ADAM9.

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absence of ADAM17. In addition, we found that the lack of ADAM17 did not affect mRNA levels of iRhom2 in LPS-treated NLDM. Finally, the catalytic activity of ADAM17 is most likely not required for the stabilization of iRhom2, because the general metallloprotease inhibitor marimastat had no detectable effects on the levels of iRhom2. Further studies will be necessary to better understand the mechanism of how the stability of iRhom2 is regulated in the absence of ADAM17. Taken together, these results suggest that the presence of the ADAM17 is required for the stabilization of iRhom2. Conversely, because only the mature form of ADAM17 is affected by the lack of iRhom2 in BMDM (24, 25) or NLDM, whereas the pro-form is not, these results suggest that pro-ADAM17 is produced at similar levels in the presence or absence of iRhom2 and does not require iRhom2 for stabilization.

The observation that pro-ADAM17 can exist in the absence of iRhom2 but requires iRhom2 to be converted into its mature, processed, and EndoH-resistant form supports a model in which pro-ADAM17 assembles with newly synthesized iRhom2 in the ER, allowing both to exit the ER and enter the secretory pathway together. The pro-domain of ADAM17 is then removed by pro-protein convertases in the trans-Golgi network (37, 46). This is a prerequisite for the activity of ADAM17, which can be rapidly enhanced by many different stimuli once ADAM17 has been processed by pro-protein convertases (19, 46). The inability to detect iRhom2 in the absence of ADAM17 in NLDM and in embryos and the very low levels of iRhom2 in A17−/− mEFs suggest that it is unstable without its binding partner. Moreover, because pro-ADAM17 is stable in the absence of iRhom2, this suggests that iRhom2 can associate with pre-existing molecules of ADAM17 to form an iRhom2/ADAM17 complex, presumably after pro-ADAM17 has been translocated across the ER membrane.

iRhom2 also interacts with another multimembrane-spanning protein, STING, which is involved in regulating innate immunity to DNA viruses (35). However, we found that STING is not essential for the stabilization of the iRhom2/ADAM17 complex. In addition, iRhom2 also interacts with several cytoplasmic molecules, including members of the 14-3-3 family of signaling adapters and FRMD8/iTAP (30–33). Moreover, because iRhom2 determines the substrate selectivity of ADAM17, the iRhom2/ADAM17 complex most likely also interacts with its substrates. This notion is further supported by the observation that the phenotype caused by the “curly-bare” (cub) mutation in the cytoplasmic domain of iRhom2 (curly hair and bare skin) is only seen in the presence of the iRhom2/ADAM17 substrate amphiregulin and not in mice lacking amphiregulin (47, 48). Presumably, the interaction between WT iRhom2/ADAM17 and its substrates is transient, so as to allow rapid substrate turnover upon activation of ADAM17.

Similar to iRhom2, the related iRhom1 can be detected on the surface of mouse embryonic fibroblasts. However, unlike iRhom2, the levels of iRhom1 are not significantly affected in the absence of ADAM17, suggesting that it interacts with ADAM17 differently from iRhom2 and can be stable on its own. The migration of the biotinylated iRhom1 or iRhom2 on SDS-PAGE was comparable with that of their nonbiotinylated counterparts that could be detected by Western in whole lysates, suggesting that the iRhom1 and -2 proteins do not undergo substantial proteolytic processing en route through the secretory pathway to the cell surface. It will be interesting to characterize the interaction between iRhom1 and ADAM17 in more detail in the future.

In summary, our results provide the first evidence that endogenous iRhom2 is stabilized by the presence of ADAM17, whereas iRhom1 is not. Nevertheless, both iRhom2 proteins can be detected on the cell surface of WT cells, where they presumably interact with ADAM17. Interestingly, a second interacting partner of iRhom2, STING, is not required for the stability of iRhom2. These findings support a model in which ADAM17 is a principal partner or client of iRhom2 and in which both must be present to support the maturation and function of the iRhom2/ADAM17 complex on the cell surface.

### Experimental procedures

#### Reagents and antibodies

All reagents were purchased from Sigma–Aldrich unless specified otherwise. The rabbit antibodies against the cytoplasmic domain of iRhom2 were generated by immunizing female New Zealand White rabbits with a GSH S-transferase fusion protein with the cytoplasmic domain of mouse iRhom2 (amino acid residues 1–376, ProSci Inc., Poway, CA). The rat anti-iRhom1 and anti-iRhom2 monoclonal antibodies were generated using standard procedures (49). The iRhom1 antibody (RHF1A 20A8; IgG2a) was generated by immunization of ovalbumin-coupled peptide MSEARRDSTSSLQRKKPPW. For the anti-iRhom2 antibody (RHF2B 11H7; IgG2a) ovalbumin-coupled peptide GDWEGKQRWHRSSL was used. Antibodies against the cytoplasmic domain of mouse ADAM9 and ADAM17 have been described previously (37, 50). Antibodies against A10 were from Abcam (Cambridge, MA) (catalog no. 1244695); antibodies against STING and antibodies against mouse tubulin were from Cell Signaling (Danvers, MA); and antibodies against GAPDH were from ABClonal (Woburn, MA). Anti-LC3 was from Novus Biological (Littleton, CO) (catalog no. Nb100-2220), and anti-ubiquitin clone P4G7 was from Biologend (San Diego, CA) (catalog no. 838703). The deglycosylation enzymes EndoH and PNGase F were from New England Biolabs (Ipswich, MA). EZ-Link Sulfo-NHS-LC-Biotin was from Thermo Fisher Scientific (Waltham, MA) (catalog no. 21335). Streptavidin-Sepharose 4B beads were from Thermo Fisher Scientific (catalog no. 434341). Sigma–Aldrich was the source for chloroquine (catalog no. C6628), MG132 (catalog no. 474790), bafilomycin (catalog no. B1793), and eeyarestatin (catalog no. E1286). The inhibitors of protein degradation or endocytosis were used at the following concentrations and for the times indicated: MG132 at 10 μM for 18 h (33), chloroquine at 100 μM for 18 h (33), bafilomycin at 100 nM for 18 h (32), and eeyarestatin at 10 μM for 18 h (43). Marimastat was a gift from Dr. Ouathek Ouerfelli (Memorial Sloan Kettering Cancer Center, New York) (51).

#### Mouse lines

*iRhom2−/−* mice and *Adam17−/−* mice have been described previously (5, 24). *Sting−/−* mice were kindly provided by Dr. Liang Deng (Memorial Sloan Kettering Cancer Center) (52,
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... All animal experiments were approved by the Institutional Animal Use and Care Committee of the Hospital for Special Surgery and Weill Cornell Medicine.

**Isolation and generation of mouse primary macrophages**

To generate primary BMDM, we harvested and cultured the bone marrow cells of 4-week-old mice (equal distribution of male and female mice) as described previously (17). Briefly, femurs and tibiae were flushed with Hanks’ balanced salt solution, and washed cells were plated on Petri dishes in RPMI medium supplemented with 20% fetal calf serum and murine macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ) at 10 ng/ml. After 7 days, macrophages were collected with a plastic tissue culture scraper, plated on fresh plates at 1 × 10⁶ cells/10 cm², and stimulated with 10 ng/ml LPS for 14–18 h. For the preparation of NLDM from mice at postnatal day 1 (P1), livers were removed after euthanasia and then dispersed through a 70-μm cell strainer (Denville Scientific, Holliston, MA). Red blood cells were removed with red blood cell lysis buffer (Sigma–Aldrich) according to the manufacturer’s protocol, and cells were then cultured as described above. For 5 days in culture, 2 × 10⁶ differentiated NLDM were stimulated with 10 ng/ml LPS for 14 h and processed for Western blot analysis.

**Culture of mouse embryonic fibroblasts**

Generation and culture of immortalized mouse embryonic fibroblasts used in this study has been described previously (5, 27, 29, 54).

**Membrane preparations and lectin purification**

For the preparation of membrane protein extracts from BMDM or NLDM, cells were washed twice with PBS and then scraped directly in membrane buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA) containing protease inhibitors plus a 5 mM concentration of the zinc-binding chelator 1,10-phenanthroline (24, 37). For membrane preparations from newborn mice (P1), the animals were euthanized, and the remaining tissues were minced with razor blades and then mechanically dispersed and subjected to two 30-s treatments with a Polytron homogenizer (Kinematica, Switzerland) in membrane buffer. The cell suspension or the homogenized tissues were then treated with 30 homogenization cycles in a Dounce homogenizer (Potter–Elvehjem, Sigma–Aldrich). The cell or tissue suspension was subjected to a low-speed spin at 720 × g for 10 min at 4 °C. The supernatant was subsequently transferred to a 13-ml ultracentrifuge tube (Ultra-Clear tubes, Beckman Coulter, Brea, CA) adjusted to 12.5 ml with membrane buffer and centrifuged for 1 h at 100,000 × g in a Beckmann Optima X Ultracentrifuge in an SW40 rotor. The resulting membrane pellets were resuspended by boiling for 5 min in 1 × SDS-sample-loading buffer, separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes (see below), and then probed with antibodies against ADAM10, ADAM17, or iRhom2.

**Western blot analysis**

To generate Western blots for iRhom2 and ADAM17 from BMDM samples from iRhom2−/− mice and WT controls (Fig. 1A) extracts from 1 × 10⁶ cells were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Pall Corp., Port Washington, NY). The Western blot analyses in Figs. 1B and 3 (A–C) were performed using extracts from 100,000 × g membrane preparations (see above). Western blots of lysates of mouse embryonic fibroblasts isolated from WT, iRhom1−/−, iRhom2−/−, and iRhom1/2−/− double knockout or A17−/− knockout E14.5 embryos were performed as described previously (27). After blocking in 5% nonfat milk at room temperature for 1 h, the nitrocellulose membranes were incubated in primary antibody overnight at 4 °C (1:2000 dilution for rabbit anti-iRhom2-cyto) or for 1 h at room temperature (rat monoclonal anti-iRhom1 and anti-iRhom2 antibodies at 1:10 culture supernatant dilution). STING antibodies (Cell Signaling, Danvers, MA) were each incubated overnight at 4 °C at 1:1000 dilution. The nitrocellulose membranes were then washed three times in PBS, 0.05% Tween 20 and then incubated in either HRP-labeled goat anti-rabbit secondary antibody, HRP-labeled goat anti-mouse secondary antibody (1:5000; Promega, Madison, WI), or HRP-labeled goat anti-rat secondary antibody (1:5000; Sigma). Bound antibodies were detected using the ECL system (Thermo Fisher Scientific) and a Chemdoc image analyzer (Bio-Rad), and the images were assembled using Microsoft Powerpoint software. Loading controls were generated either by Western blotting parallel samples or by stripping membranes for 15 min at 55 °C in stripping buffer (2% SDS, 50 mM 2-mercaptoethanol in 62 mM Tris, pH 6.7). These membranes were blocked as described and then incubated with anti-ADAM9, anti-ADAM10, anti-GAPDH, or anti-tubulin antibodies at 1:2000, and the bound antibodies were detected as described above.

**Cell-surface biotinylation**

Mouse embryonic fibroblasts or differentiated BMDM that had been left untreated or had been treated with 10 ng/ml LPS were cell surface–biotinylated by incubation with a 1 mg/ml solution of the non-membrane-permeable biotinylation reagent EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) in sterile PBS for 45 min at 4 °C. The reaction was quenched with 0.1 M glycine in PBS, and then the cells were lysed in cell lysis buffer (PBS, 1% Triton X-100 containing protease inhibitors plus a 5 mM concentration of the zinc-binding chelator 1,10-phenanthroline (24, 37) and 5 μM marimastat).
Extracts containing biotinylated proteins were incubated with 10 µl of washed streptavidin-Sepharose 4B beads for 30 min at 4°C. The beads were then washed four times with cell lysis buffer, and bound proteins were eluted in boiling SDS-sample buffer and separated on NuPAGE Novex 3–8% Tris acetate protein gels (Thermo Fisher Scientific) and then subjected to Western blot analysis with antibodies against iRhom1 or -2, ADAM17, or ADAM9, as indicated. All other samples were separated on 10% SDS-polyacrylamide gels.

**RT-qPCR analysis**

Total RNA from cultured WT, Adam17−/−, or iRhom2−/− NLDM or from WT, iRhom2−/−, or Sting−/− BMDM was isolated with RNeasy (Qiagen, Germantown, MA) and subsequently reverse-transcribed (Oligo-dT/Superscript RT III, Qiagen, Germantown, MA). Oligonucleotides for iR2 and GAPDH were purchased from Qiagen. Sequences for β-actin oligonucleotides were as follows: β-actin forward, 5’-AGGTGTGCGACTTTTATTGGTCTCAA-3’; β-actin reverse, 5’-TGTGATGAAAGCTTTGGTCTCACTTTTATTGGTCTCAA-3’; RT-qPCR was performed using SYBR Green on an ABI PRISM 7900HT cycler (both from Applied Biosystems, Thermo Fisher Scientific). GAPDH was used as an endogenous control to normalize each sample. Three independent experiments were performed in triplicate.

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