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Mammalian EGF receptor activation by the rhomboid protease RHBDL2

Colin Adrain[1], Kvido Strisovsky[1], Markus Zettl[1], Landian Hu[2], Marius K. Lemberg[3] & Matthew Freeman[1+]

[1]Medical Research Council Laboratory of Molecular Biology, Cambridge, UK, [2]Molecular Genetics Lab, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, and [3]Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany

The epidermal growth factor receptor (EGFR) has several functions in mammalian development and disease, particularly cancer. Most EGF ligands are synthesized as membrane-tethered precursors, and their proteolytic release activates signalling. In *Drosophila*, rhomboid intramembrane proteases catalyse the release of EGF-family ligands; however, in mammals this seems to be primarily achieved by ADAM-family metalloproteases. We report here that EGF is an efficient substrate of the mammalian rhomboid RHBDL2. RHBDL2 cleaves EGF just outside its transmembrane domain, thereby facilitating its secretion and triggering activation of the EGFR. We have identified endogenous RHBDL2 activity in several tumour cell lines.

Keywords: cancer; EGF receptor; intramembrane protease; mammal; rhomboid

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INTRODUCTION

The epidermal growth factor receptor (EGFR) has an important function in animal development, and its hyperactivity contributes to many types of human cancer (Salomon *et al*, 1995; Blobel, 2005). Most EGFR ligands are synthesized in a latent, membrane-tethered form; subsequent proteolytic cleavage is required to release the extracellular domain as a diffusible growth factor (Blobel *et al*, 2009). In *Drosophila*, the rhomboid family of intramembrane serine proteases catalyses the cleavage of membrane-tethered epidermal growth factor (EGF)-like ligands (Lee *et al*, 2001; Urban *et al*, 2001; Freeman, 2008). A rhomboid is also implicated in the proteolytic release of the *Caenorhabditis elegans* EGF-like ligand Lin-3 (Dutt *et al*, 2004). Although many aspects of EGFR signalling are conserved, no evidence so far implicates rhomboid proteases in the release of mammalian EGF-like ligands (Lohi *et al*, 2004; Pascall & Brown, 2004; Blobel *et al*, 2009). By contrast, substantial evidence, including from studies of knockout mice, implies that proteolytic release is triggered by several members of the ADAM (a disintegrin and metalloproteinase) metalloprotease family (Peschon *et al*, 1998; Blobel, 2005; Blobel *et al*, 2009).

Mammalian EGF is synthesized as a large (170 kDa) precursor containing eight EGF repeats as well as the bioactive EGF module (Fig 1A; Carpenter & Cohen, 1979). The reported molecular weight of secreted EGF is highly variable. For example, in the rodent submaxillary gland, EGF is secreted as a 6-kDa form (comprising only the bioactive EGF module), whereas in other contexts, such as the kidney and mammary gland, higher-molecular-weight soluble species in the range of 140–170 kDa—comprising most of the molecule—are observed (Mroczkowski *et al*, 1988; Jorgensen *et al*, 1994; Dempsey *et al*, 1997). This variety in weight is reflected in several proteases proposed to be involved in the cleavage and trimming of EGF in different contexts, including not only ADAMs (Dempsey *et al*, 1997; Sahin *et al*, 2004), but also kallikreins (Jorgensen *et al*, 1994), arginine esterases (Breyer & Cohen, 1990) and an unidentified membrane-anchored serine protease (Le Gall *et al*, 2004).

In this study, we focus on the potential involvement of mammalian rhomboid proteases in EGF ligand secretion. We find that, in contrast to earlier reports, EGF is a substrate for the mammalian rhomboid RHBDL2 (rhomboid-like 2). This provides the first direct implication of the involvement of mammalian rhomboid proteases in EGFR signalling. We also show that endogenous RHBDL2 activity triggers EGF secretion in a panel of tumour cell lines, independent of metalloproteases. Our data indicate that, similarly to in *Drosophila*, rhomboids can have a role in the generation of bioactive EGF ligands in mammals.

RESULTS AND DISCUSSION

Cleavage of EGF by mammalian rhomboid RHBDL2

To test whether mammalian rhomboids can cleave EGF ligands, we screened a panel of these growth factors in a cell-based secretion assay (Fig 1; Table 1). We used the metalloprotease inhibitor BB94 (batimastat) in this and subsequent experiments

[1]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK
[2]Molecular Genetics Lab, Institute of Health Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences & Shanghai Jiao Tong University School of Medicine, 225 Chongqing Nan Road, Shanghai 200025, China
[3]ZMBH, DKFZ–ZMBH Allianz, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany
[+]Corresponding author. Tel: +44 1223 402351; Fax: +44 1223 412142;
E-mail: mf1@mrc-lmb.cam.ac.uk

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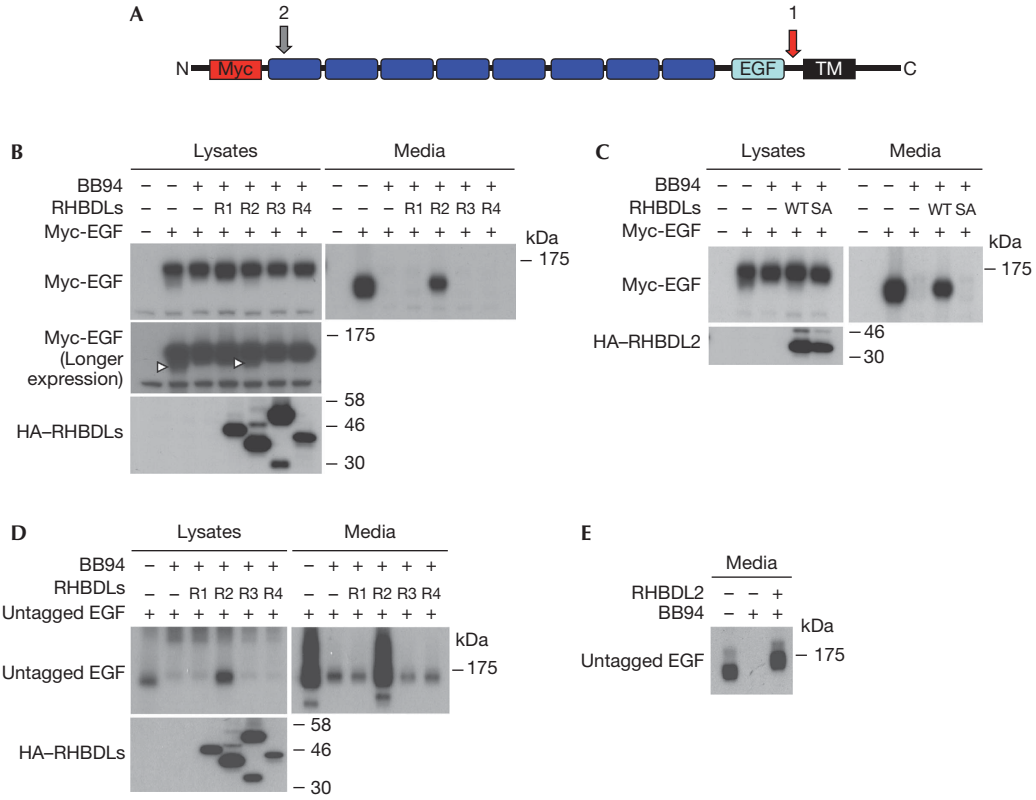

Fig 1 | Epidermal growth factor cleavage by RHBDL2. (**A**) EGF domain structure and position of the Myc tag. Red arrow: primary cleavage event by rhomboid or metalloproteases; grey arrow: putative amino-terminal trimming by metalloproteases. (**B–E**) Secretion assay in COS7 cells transfected with rhomboids plus (**B**,**C**) Myc-tagged or (**D**,**E**) untagged EGF. (**B**) Western blots show that RHBDL2 (R2), but not other mammalian rhomboids (R1, R3, R4), can cleave EGF. Expression of HA-tagged rhomboids is shown below. (**C**) A serine to alanine (SA) catalytic mutant of RHBDL2 does not cleave EGF. (**D**) Untagged EGF is a substrate for RHBDL2. The faint bands in lanes 2, 3, 5 and 6 are background EGF cleavage caused by incomplete ADAM inhibition. (**E**) Secreted EGF cleaved by RHBDL2 runs more slowly than metalloprotease-cleaved EGF. Samples were resolved on an 8% SDS-tris-glycine gel. EGF, epidermal growth factor; HA, haemagglutinin; RHBDL, rhomboid-like 2; WT, wild type.

to inhibit background cleavage by metalloproteases. As shown in Fig 1B, we found that the mammalian rhomboid RHBDL2 cleaved Myc-tagged EGF, leading to its secretion into the culture medium. Some RHBDL2-cleaved EGF was also detected in cell lysates (Fig 1B). Consistent with previous studies (Mroczkowski *et al*, 1988; Jorgensen *et al*, 1994; Dempsey *et al*, 1997), EGF was detected as a high-molecular-weight species of approximately 150–160 kDa, rather than the maximally processed 6-kDa form that is normally observed in the rodent submaxillary gland (Fig 1). A catalytically inactive version of RHBDL2 was unable to trigger EGF secretion, confirming that processing required rhomboid proteolytic activity (Fig 1C). An untagged version of EGF was an equally efficient substrate for RHBDL2. However, the EGF antibody—which is specific to the bioactive (most carboxy-terminal) EGF domain (see schematic in Fig 1A) and requires non-reducing SDS–polyacrylamide gel electrophoresis conditions—could not detect the transmembrane precursor of EGF, and instead only detected cleaved EGF species within the lysate (Fig 1D). Untagged EGF was used in all subsequent experiments in this study.

The RHBDL2-cleaved species of untagged EGF (which was generated under conditions in which metalloproteases were inhibited) was several kilodaltons larger than its metalloprotease-generated counterpart (Fig 1E). As the RHBDL2 cleavage site in EGF (see below) is located within two amino acids of the site that has been determined for metalloprotease-cleaved EGF (Arg 1029; Savage *et al*, 1972), this does not explain the observed difference in electrophoretic mobility. We infer that the difference in molecular weight must be a consequence of trimming within the amino-terminus by a BB94-sensitive metalloprotease (Fig 1A). This size difference allows us to unambiguously resolve secreted EGF originating from ADAM, rather than RHBDL2 cleavage.

Specificity of RHBDL2 for EGF

To determine the specificity of RHBDL2 for EGF, we tested the ability of RHBDL2 to cleave a panel of control type I transmembrane proteins and several other EGF ligands including some neuregulins (supplementary Fig S1 online). No other protein was substantially cleaved (Table 1; supplementary Fig S1 online), suggesting that proteolysis of EGF by RHBDL2 is specific. We cannot rule out other substrates, but we conclude that RHBDL2 is not a highly promiscuous transmembrane domain protease, and that it does not cleave all EGF-like ligands.

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Table 1 | Proteins tested for cleavage by RHBDL2

Molecule	Accession number	Cleavage by RHBDL2
EGF	NM_010113.2	+++
TGFα	NM_003236	–
Epiregulin	NM_007950.2	–
Betacellulin	NM_007568.3	–/+
Amphiregulin	NM_009704.3	–
HB–EGF	NM_001945.2	–
Vaccinia virus growth factor	AY243312.1	–
TMEFF2	NM_019790.3	–
Calnexin	NM_001746.3	–
TACE	NM_009615.5	–
S1P	NM_019709.3	–
NDF β4a (Nrg1 isoform)	U02322.1	–
Rat glial cell growth factor (Nrg1 isoform)	AY995221.1	–/+
Nrg4	NM_138573.2	–

EGF, epidermal growth factor; HB, heparin binding; NDF, neu differentiation factor; S1P, site-1 protease; TGF, transforming growth factor; TMEFF2, transmembrane protein with EGF-like and two follistatin-like domains 2.

EGF and RHBDL2 colocalize at the plasma membrane

Green fluorescent protein-tagged RHBDL2 and Myc-tagged EGF were transfected into HeLa cells, which express endogenous RHBDL2 message. To minimize the risk of overexpression artefacts, we limited our analysis to cells expressing low levels of the labelled proteins. Both RHBDL2 and EGF colocalized extensively in the plasma membrane and in filopodial-like projections (Fig 2). In cells expressing the transgenes at higher levels they also accumulated earlier in the secretory pathway (data not shown).

In vitro determination of rhomboid cleavage site in EGF

To further explore the role of rhomboid in EGF biogenesis, we determined the RHBDL2-catalysed cleavage site of EGF. We used a combined *in vitro* and cell-based approach. The cleavage site was determined *in vitro* with a bacterial rhomboid (mammalian rhomboids work inefficiently *in vitro*; Lemberg *et al*, 2005); this site was then confirmed in mammalian cells.

Previous work has shown that a variety of prokaryotic and eukaryotic rhomboids recognize similar substrate motifs (Strisovsky *et al*, 2009). RHBDL2 behaved similarly to other rhomboids: it cleaved the model substrate Spitz, and mutation of the residue in the P1 position (that is, immediately N-terminal to the scissile bond; Ala 138) to phenylalanine completely blocked cleavage (Fig 3A). In addition, mutation of the P4 or P2′ residues to glycine (Strisovsky *et al*, 2009) impaired cleavage and these mutations in combination completely blocked cleavage (Fig 3A). These data indicated that RHBDL2 recognizes a similar motif in substrates as previously reported rhomboids, including AarA and GlpG from *Providencia stuartii* and *Escherichia coli*, respectively (Strisovsky *et al*, 2009). We therefore reasoned that we could use these bacterial rhomboids to predict the cleavage site in EGF.

Adapting a previous strategy (Strisovsky *et al*, 2009), we used a chimeric substrate, comprising the juxtamembrane and transmembrane regions of EGF, fused to maltose-binding protein and thioredoxin (Fig 3B). AarA and GlpG, but not their catalytically inactive mutants, generated a 27-kDa cleavage product, the molecular weight of which was consistent with cleavage within or near the EGF transmembrane domain (Fig 3B; data not shown). Analysis of these cleavage products by N-terminal sequencing showed that the neo-N-termini generated by both AarA and GlpG were identical and implicated Ala 1031–Gly 1032—in the region just N-terminal to the predicted transmembrane domain—as the scissile bond (Fig 3B,C). The cleavage site was within a stereotypic recognition motif (Strisovsky *et al*, 2009) containing hydrophobic, bulky residues in the P4 and P2′ positions and a small amino acid (Ala) in the P1 position (Fig 3C).

In vivo cleavage of EGF by RHBDL2

We sought to confirm *in vivo* the *in vitro*-mapped EGF cleavage site. The EGF juxtamembrane region and the upper part of the transmembrane domain has three potential cleavage sites: Ala 1031–Gly 1032 (identified by the *in vitro* experiments), Ala 1043–Val 1044 or Ala 1048–Leu 1049, which were both also flanked by permissive P4 and P2′ residues (Fig 3C). Each of these candidate P1 residues was mutated to phenylalanine, but only mutation of Ala 1031 blocked proteolysis by overexpressed RHBDL2 in COS7 cells. By contrast, this mutant retained susceptibility to ADAM-mediated shedding, confirming that its biosynthesis and transport was normal (Fig 3D). As a further control, we mutated several other small residues (Gly 1032, Gly 1034 and Cys 1045) that were possible P1 candidates (although not framed by permissive P4 and P2′ residues), but none affected RHBDL2 cleavage of EGF (Fig 3D). To confirm the cleavage position at Ala 1031, we mutated the hydrophobic residues in the predicted P4 and P2′ positions to glycine. As expected, both specifically impaired RHBDL2-dependent proteolysis of EGF, especially when combined as a double P4/P2′ mutant (Fig 3E). Taken together, these data confirm that, *in vivo*, RHBDL2 cleaves EGF within the juxtamembrane region at Ala 1031, as predicted from our *in vitro* experiments. This explains the discrepancy between our results and an earlier study, in which a chimera containing the EGF transmembrane domain without the relevant juxtamembrane sequences was not cleaved (Pascall & Brown, 2004).

Endogenous RHBDL2 activity in tumour cell lines

There are no effective RHBDL2 antibodies; we therefore used quantitative PCR on a panel of tumour cell lines to identify cells that express endogenous RHBDL2. RHBDL2 expression was not ubiquitous, but was present in several cell lines, including HeLa, Pnt1A, U1752, T47D and B2B (Fig 4A). By contrast, RHBDL2 expression was low or absent in 293T, SW480, HCT116, MB453 (Fig 4A) and COS7 cells (data not shown). This observation provided the basis on which to test the hypothesis that endogenous RHBDL2 activity might contribute to EGF secretion. We transduced cells with EGF-expressing lentiviruses at a multiplicity of infection of less than one copy per cell, and examined the secretion of EGF into the culture media from the resultant stable cell lines. To measure metalloprotease-independent EGF

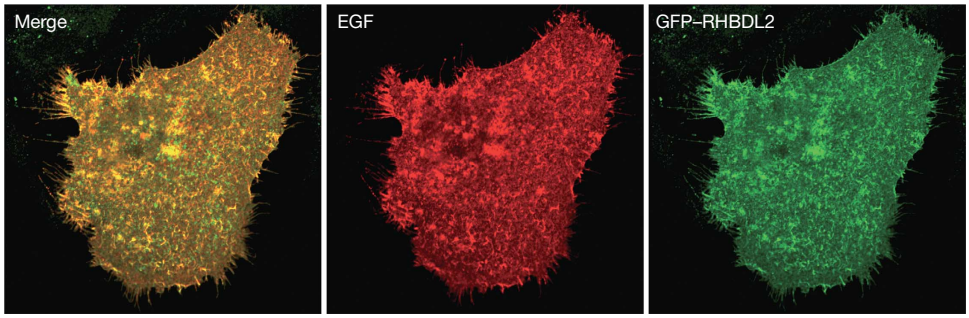

Fig 2 | Colocalization of green fluorescent protein (GFP)–RHBDL2 and epidermal growth factor (EGF). A representative ×40 confocal image is shown.

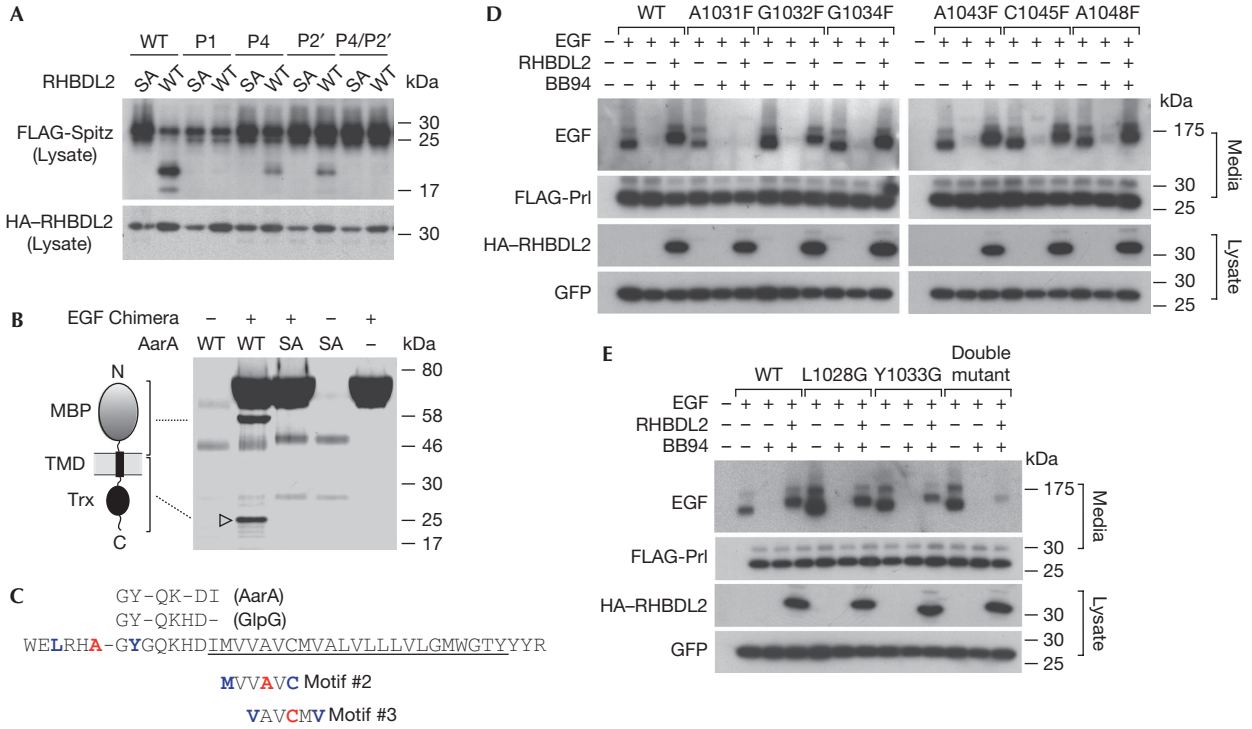

Fig 3 | Identification of the cleavage site in EGF. (**A**) Western blot showing intracellular cleavage of Spitz and its recognition motif mutants by RHBDL2–KDEL in COS7 cells. HA–RHBDL2–KDEL expression is shown below. (**B**) *In vitro* cleavage of an MBP-EGF-Thioredoxin chimera by WT or SA mutant AarA. A schematic representation of the chimera is included. (**C**) The neo-amino-termini of AarA and GlpG-cleaved EGF, identified by N-terminal sequencing. The P1 residue Ala 1031 is shown in red, P4 and P2′ residues are shown in blue, and the TMD is underlined. Two other putative recognition motifs are indicated. (**D**) Mutation of the P1 residue (Ala 1031) blocks EGF secretion. FLAG-prolactin and GFP are secretion and transfection controls. (**E**) Combined mutations of the P4 (Leu 1028) and P2′ (Tyr 1033) residues in EGF impair its RHBDL2-induced secretion. EGF, epidermal growth factor; GFP, green fluorescent protein; HA, haemagglutinin; MBP, myelin basic protein; RHBDL2, rhomboid-like 2; SA, serine to alanine; TMD, transmembrane domain; WT, wild type.

secretion, we compared the release of EGF in the absence and presence of BB94. In cells expressing little or no RHBDL2, most EGF secretion was dependent on metalloprotease activity (Fig 4B). By contrast, BB94 failed to block secretion in cells that expressed significant levels of endogenous RHBDL2 (Fig 4C). When metalloproteases were inhibited in these RHBDL2-expressing cells, a higher-molecular-weight species of EGF was secreted, similar to the form generated by RHBDL2 overexpression in COS7 cells (Fig 4C). These data indicate that endogenous RHBDL2 activity is responsible for metalloprotease-independent shedding of EGF (Fig 4C). In support of this conclusion, when HeLa cells were transfected with the EGF mutants engineered to be uncleavable by RHBDL2 (Fig 3D,E; expressed at equivalent levels to the wild-type proteins), metalloprotease-independent cleavage was blocked (Fig 4D). Importantly, RHBDL2 was also able to cleave EGF in the absence of BB94 (supplementary Fig S2 online), demonstrating that RHBDL2 cleavage of EGF is not triggered indirectly by treatment of cells with BB94.

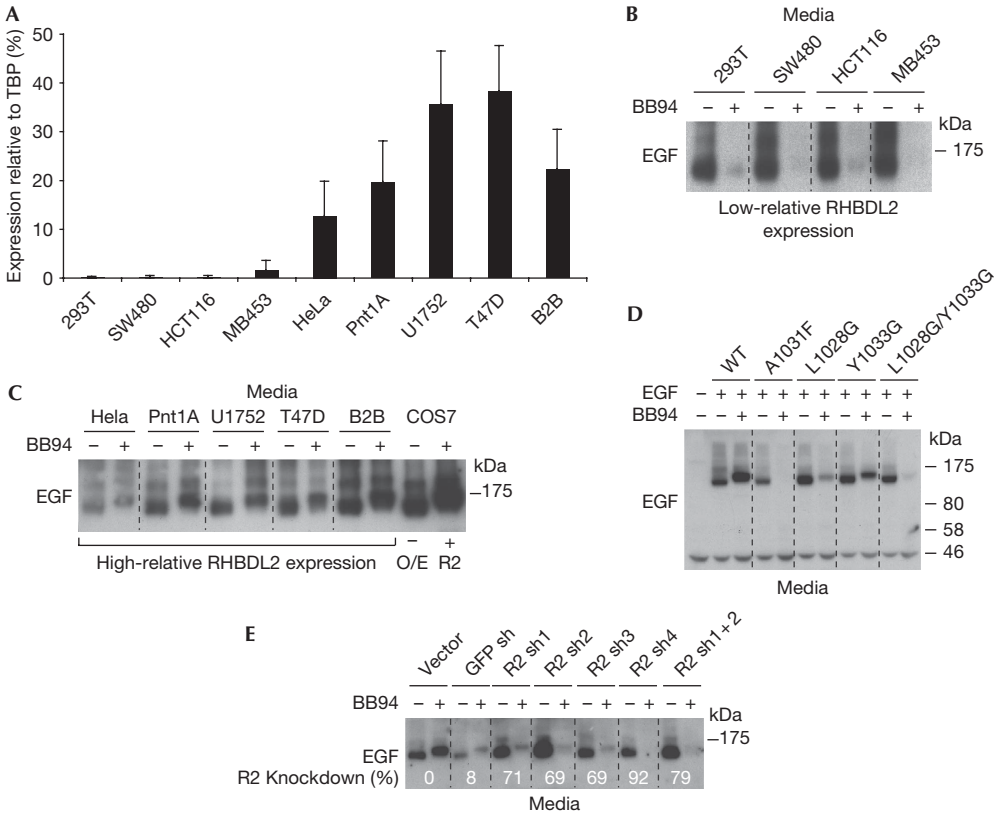

Fig 4 | Endogenous RHBDL2 expression and activity. (**A**) RHBDL2 messenger RNA expression in tumour cell lines assessed by quantitative PCR, expressed relative to a control gene for TBP. The mean ± s.d. from three replicate experiments is shown. (**B**) EGF secretion in cell lines that express little or no RHBDL2 is blocked by BB94. (**C**) Metalloprotease-independent EGF secretion in cells that have high levels of endogenous RHBDL2. The cell lines shown in the first ten lanes were transduced with EGF lentivirus. EGF secretion ± BB94 was determined by immunoblotting. The last two lanes are a positive control for the mobility of RHBDL2-cleaved EGF and represent supernatants from COS7 cells transfected with EGF without RHBDL2, minus BB94 (second last lane) or with RHBDL2 plus BB94 (last lane). (**D**) Impaired metalloprotease-independent secretion of EGF recognition motif mutants in HeLa cells, which express endogenous RHBDL2. (**E**) Impaired EGF secretion on RNA interference of RHBDL2 in EGF-expressing HeLa cells, which were transduced with shRNAs for vector, GFP or four non-overlapping RHBDL2-specific shRNA lentiviruses. The percentage knockdown of RHBDL2 mRNA relative to the vector control is indicated. EGF, epidermal growth factor; GFP, green fluorescent protein; RHBDL2, rhomboid-like 2; shRNA, short-hairpin RNA; TBP, TATA box-binding protein; WT, wild type.

RHBDL2 regulates ADAM-independent EGF secretion

EGF-expressing stable HeLa cells were transduced with a panel of lentiviruses expressing four non-overlapping RHBDL2-specific short-hairpin RNAs (shRNAs; see Methods section). As shown in Fig 4E, all four shRNAs triggered substantial knockdown of endogenous RHBDL2 expression. We then examined their ability to modulate EGF secretion, by making a pairwise comparison of EGF secretion with or without BB94. Metalloprotease-independent EGF secretion was significantly impaired in cells transduced with any of the four RHBDL2 shRNAs (Fig 4E). By contrast, RHBDL2 ablation did not impair metalloprotease-dependent EGF shedding, suggesting that RHBDL2 and ADAMs participate in separate pathways rather than acting serially. Consistent with this model, EGF secretion in these cells was effectively blocked when metalloproteases were inhibited in conjunction with RHBDL2 knockdown by RNA interference (Fig 4E). Taken together with the cleavage site mutant experiments shown in Fig 4D, these data indicate that some cell types have parallel pathways for EGF secretion; one of which is controlled by metalloproteases and the other mediated by RHBDL2.

RHBDL2 can trigger EGFR activation

To test whether RHBDL2-cleaved EGF can activate the EGFR, we exposed serum-starved A431 cells—which express high levels of the EGFR—to supernatants from cells expressing RHBDL2 and EGF, in the presence of BB94. When ADAMs were inhibited, RHBDL2 was able to drive EGFR activation (Fig 5A,B). Quantitative analysis showed that ADAMs and rhomboid triggered EGFR activation in a dose-dependent manner, with similar efficiency (Fig 5C). Overall, these data indicate that RHBDL2 can trigger EGFR activation by metalloprotease-independent secretion of EGF and support the idea that RHBDL2 can drive a parallel pathway for EGFR activation in mammalian cells. However, in the absence of genetic experiments, the physiological significance of this remains to be established.

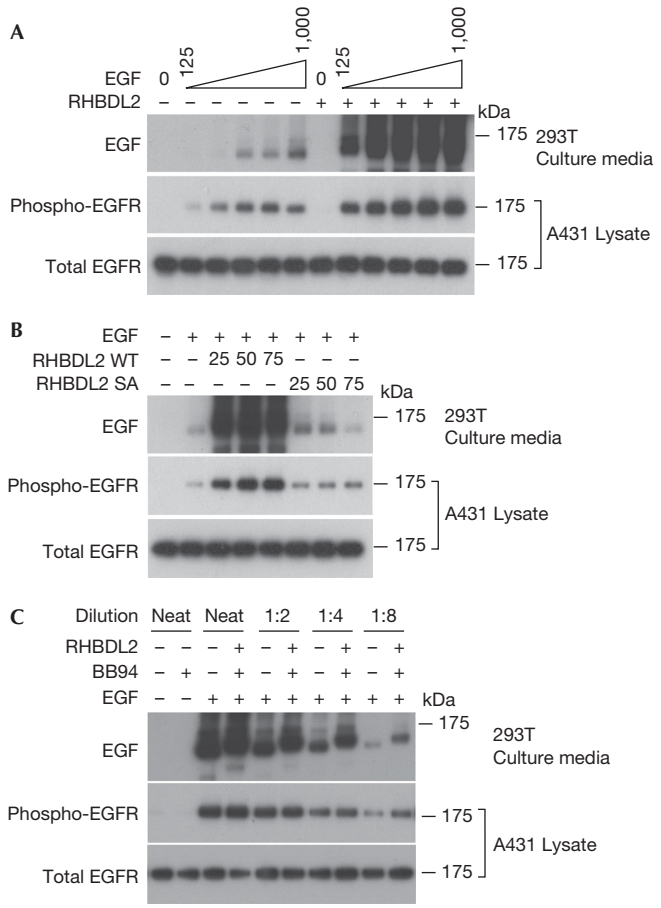

Fig 5 | EGFR activation by RHBDL2. HEK 293T cells were transfected with (**A**) untagged EGF (125, 250, 500, 750 and 1,000 ng) plus 50 ng of RHBDL2 or (**B**) 250 ng of EGF plus the indicated amounts of RHBDL2 WT or SA mutant. All treatments were performed in the presence of 10-μM BB94. Cell supernatants were assayed on A431 cells. The upper panels are anti-EGF western blots of the culture supernatants. The middle and lower panels are A431 cell lysates probed for phospho- or total EGFR, respectively. (**C**) HEK 293T cells were transfected with EGF ± RHBDL2. Supernatants were generated with ± BB94 and assayed on A431 cells. EGFR, epidermal growth factor receptor; HEK, human embryonic kidney; RHBDL2, rhomboid-like 2; SA, serine to alanine; WT, wild type.

In what biological context might RHBDL2 be responsible for EGF release? On the basis of non-cleavable mutants and shRNA experiments, our data indicate that metalloprotease and rhomboid activity might regulate parallel pathways for EGF secretion. Alternatively, EGFR signalling might exclusively rely on either metalloproteases or rhomboids in different contexts. In support of this, and as mentioned above, several proteolytic activities have been reported to account for EGF secretion in a variety of biological contexts (Breyer & Cohen, 1990; Jorgensen *et al*, 1994; Dempsey *et al*, 1997; Le Gall *et al*, 2004; Sahin *et al*, 2004). The only genetic evidence that so far implicates a protease in EGF secretion is from ADAM-10-null embryonic fibroblasts (Sahin *et al*, 2004). However, whether ADAM10 has a general role in EGF secretion in other tissues is unclear, because ADAM10-null mice die during embryogenesis (Hartmann *et al*, 2002). Public domain messenger RNA expression profiles for RHBDL2 indicate that, as in the cancer cell lines we assayed, it is not expressed ubiquitously but is restricted to a few tissues, including intestine, stomach, prostate, bladder and skin (http://www.biogps.gnf.org/). As EGF and the EGFR are also expressed in the same places, they are candidates for tissues in which RHBDL2 might have a tissue-specific role.

Beyond speculation about the physiological role of rhomboid processing of EGF, the demonstration that RHBDL2 can activate EGFR signalling by triggering EGF secretion could have relevance to oncogenic mechanisms. Hyperactivity of the EGFR is implicated in many tumours (Salomon *et al*, 1995). Several molecular mechanisms underlie this dysregulation; of particular relevance to rhomboids, these include the autocrine activation of the receptor by unregulated release of ligands (Sporn & Roberts, 1985; Salomon *et al*, 1995). Another possible link between mammalian rhomboids and cancer is suggested by recent work showing that the inactive rhomboid-like proteins, iRhoms, can regulate EGF signalling in both *Drosophila* and mammals by promoting endoplasmic reticulum-associated degradation of EGF family ligands (Zettl *et al*, 2011). In *Drosophila,* these ligands are substrates for active rhomboids, so the iRhom effectively regulates a rhomboid-dependent process. iRhom1 (also called RHBDF1) has been reported to participate in growth and apoptosis control in several epithelial-cancer cell lines (Yan *et al*, 2008), and to participate in EGFR transactivation (Zou *et al*, 2009). Another distantly related inactive rhomboid-like protein—RHBDD2, which is not a member of the iRhom subfamily—is significantly upregulated in breast carcinomas (Abba *et al*, 2009). Although no way in which to integrate these results has yet emerged, these reports suggest possible links between rhomboids, EGFR signalling and cancer in mammals. Our discovery that RHBDL2 can activate EGFR signalling provides a new mechanistic perspective on these functional studies.

METHODS

Rhomboid-based secretion assays. Cell-based rhomboid assays were based on previously published protocols (Urban *et al*, 2001). Full details, including expression plasmid cloning, point mutagenesis and antibodies used, are described in the supplementary information online.

Determination of the bacterial rhomboid cleavage site in EGF. A chimeric protein comprising the transmembrane and juxtamembrane regions (Gln 1019 to Asn 1075) of mouse EGF was expressed in *E. coli*, affinity purified and cleaved *in vitro* by bacterial rhomboids AarA and GlpG. Full details of the *in vitro* assay (Strisovsky *et al*, 2009) are described in the supplementary information online.

EGFR activation assays. Human embryonic kidney 293T cells were transfected by standard techniques, as described in the supplementary information online. After 36 h, serum-free culture supernatants were assayed for their ability to activate EGFR on A431 cells, as described in the supplementary information online.

Lentivirus-mediated transduction. A total of 293ET cells were transfected in 10-cm plates with 6 μg of the appropriate long terminal repeat-containing expression plasmid, 4.2 μg of the packaging plasmid pCMV delta8.91 and 1.8 μg of pMD.VSVG (see supplementary information online). The following day, media

were changed. Lentivirus was collected after 48–72 h and diluted by two- to fourfold before infection of target cells, in the presence of 8-μg/ml polybrene. RHBDL2 lentiviral shRNAs were designed by the RNA interference consortium (Root *et al*, 2006).

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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