Supplemental Methods and Materials

Antibodies and immunoblotting

The following antibodies were used in this study: Anti-HA 16B12 (Covance), anti-Myc A-14 polyclonal (Santa Cruz); anti-Myc (9E10) antibodies (Sigma); rabbit anti-EGF (Millipore), anti-EGFR Phospho tyrosine 1173 Clone 9H2 (Millipore) and total EGFR Clone 20G3 (Millipore). An anti-NDF monoclonal antibody was the kind gift of Dr. Yosef Yarden. Secondary HRP-coupled antibodies were from Santa Cruz Biotech. Secondary fluorophore-coupled antibodies were from Invitrogen. HRP activity was detected by enhanced chemiluminescence (GE Healthcare).

Cell culture, transfection and rhomboid-based secretion assays

Cells were maintained in Dulbecco’s Modified Eagles Medium supplemented with 10 % foetal calf serum. For COS7-based overexpression assays, cells were first transfected in 6-well plates using Fugene 6 transfection reagent (Roche) using 250 ng of substrate and 25 ng of rhomboid expression plasmids. The total DNA amount was adjusted to 1 µg with pcDNA3.1(+) vector. Eighteen hours post-transfection, cells were washed once in PBS and the secretion assay was then performed for 30 hours in serum-free medium containing 10 µM BB-94 or with DMSO as a vehicle control. Assays using stable EGF-expressing cell lines generated following lentivirus transduction were performed similarly. The culture supernatants were then collected by centrifugation at 16,000g for 10 minutes, and proteins precipitated by adding TCA (12% w/v). Precipitated proteins were recovered by centrifugation, washed in acetone and solubilised in 30 µl 1x SDS PAGE sample buffer whereas the monolayers were lysed in 150 µl of sample buffer. 15 µl of each were then electrophoresed. Note that to resolve the difference in molecular weight between ADAM-cleaved and RHBDL2-cleaved EGF, samples were typically electrophoresed on 4-20% SDS-PAGE gels (Novagen) until a ca. 17 kDa protein marker was run to the edge of the gel, although there was some
variability in the distance that the samples were electrophoresed. In addition, the panel shown in Figure 1E is from samples electrophoresed on an 8% gel. For detection of untagged EGF using the anti-EGF antibody (Millipore), which is specific to the bioactive (i.e., most C-terminal) EGF domain (see schematic in Fig. 1A) samples were denatured under non-reducing conditions, as recommended by the manufacturer. Thus, the mobility of tagged versus untagged EGF detected under reducing versus non-reducing conditions respectively, differed slightly. Note that the EGF antibody, could not detect the transmembrane precursor of EGF and instead only detected cleaved EGF species within lysates.

Plasmids, Cloning and Point mutagenesis

Plasmids were generated according to standard cloning techniques. Unless indicated otherwise, all plasmids were constructed using pcDNA3.1 (Invitrogen). Expression plasmids for mouse RHBDL1, RHBDL2 and RHBDL3 with an N-terminal triple HA tag were described previously (Lohi et al., 2004) and mouse RHBDL4 (IMAGE 3494511) was similarly cloned with an N-terminal triple HA tag. For Fig. 3A, HA-RHBDL2 was modified to contain a C-terminal KDEL ER retention signal. Spitz (AAA28894) was cloned with a triple FLAG-tag in the N-terminus. Mouse Amphiregulin (NM_009704.3), mouse Betacellulin (NM_007568.3), mouse Epiregulin (NM_007950.2), mouse EGF (NM_010113.2) and mouse TMEFF2 (NM_019790.3) were cloned without their signal peptides into a pcDNA3.1-based expression vector containing the Spitz signal peptide fused to a triple Myc tag. Similarly, the following were cloned into an expression vector comprising the Spitz signal peptide fused to a triple FLAG tag: human prolactin (NM_000948.3), human HB-EGF (NM_001945.2), human Calnexin (NM_001746.3), mouse TACE lacking the pro-peptide (NM_009615.5), mouse S1P lacking the pro-peptide (NM_019709.3) and the vaccinia virus growth factor (AY243312.1). EGF and Spitz point mutants were generated using the Quikchange II mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions, using mouse EGF cDNA (NM_010113.2) as a template. The expression plasmids for rat
NDF β4a and rat Glial Cell Growth Factor were the kind gifts of Drs Yosef Yarden and Doug Falls.

**Immunofluorescence**

HeLa cells were plated on glass coverslips at a density of 2 x 10^5 per well the day before transfection. The following day, cells were transfected with 25 ng of N-terminally GFP-tagged RHBDL2 and 250 ng of Myc-tagged EGF. Approximately 24 hours later, cells were fixed in 3% paraformaldehyde and processed as previously described (Lohi et al., 2004). Cells were then probed with a 1:500 dilution of anti-Myc (9E10) antibodies (Sigma). As the GFP-RHBDL2 signal was faint after fixation, it was detected using a 1:5000 dilution of rabbit anti-GFP.

**Determination of bacterial rhomboid cleavage site in EGF**

A DNA segment encoding the amino acids Gln1019 to Asn1075 of mouse EGF (NM_010113.2) that encompasses the transmembrane domain and the adjacent juxtamembrane regions was cloned in frame between the KpnI and Xbal sites of pKS29 (Strisovsky et al., 2009) to generate a maltose binding protein - EGF TMD - thioredoxin fusion protein (MBP-EGFtmd-Trx). The full-length fusion protein was expressed in *E.coli* MG1655 ΔglpEGR::kan (gift of Philip N. Rather, Emory University School of Medicine, Atlanta, USA), isolated and purified by NiNTA and amylose affinity chromatography. It was then subjected to cleavage by purified bacterial rhomboids AarA (*Providencia stuartii*) and GlpG (*E.coli*) in vitro. Briefly, 20 µg of purified MBP-EGFtmd-Trx was incubated with 2 µg of rhomboid in a 20 µL reaction in the presence of 50 mM HEPES-NaOH (pH 7.5), 250 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol and 0.05% (w/v) n-dodecyl-β-D-maltoside (DDM, Glycon Biochemicals GmbH, Germany) at 37°C for 15 hrs. Cleavage products were then separated on an SDS PAGE and electroblotted onto a PVDF membrane (Immobilon-PSQ, Millipore). The cleavage site in EGF was determined by N-terminal sequencing of the C-terminal cleavage products by automated Edman
degradation using a Procise™ Protein Sequencing System (491 Protein Sequencer, PE Applied Biosystems).

Quantitative PCR (qPCR)

Total RNA was extracted from cells using the RNeasy kit (Qiagen). 1 µg of RNA was then transcribed into cDNA using the Superscript III first strand synthesis kit (Invitrogen). qPCR was performed on the resultant cDNA using Gene Expression Mastermix and a human RHBDL2 taqman probe (Hs00384848_m1) in accordance with the manufacturer’s instructions (Applied Biosystems). A probe for TBP (TATA box-binding protein) was the kind gift of Dr Mariann Bienz (de la Roche et al., 2008).

Lentivirus-mediated transduction

For expression of mouse EGF, the untagged gene was subcloned into a derivative of the expression vector pSin-CSGW. For shRNA, pLKO.1 plasmids containing human RHBDL2-specific shRNAs (clone IDs TRCN0000048598, TRCN0000048599, TRCN0000048600, and TRCN0000048601) or control hairpins from the RNAi consortium (TRC) (Root et al., 2006) were purchased from Open Biosystems. To generate VSVG-pseudotyped lentivirus, based on the three plasmid lentivirus packaging system, 293 ET cells were transfected in 10 cm plates with 6 µg of the LTR-containing expression plasmid, 4.2 µg of the packaging plasmid pCMV delta8.91 and 1.8 µg of pMD.VSVG (Naldini et al., 1996; Zufferey et al., 1997). The following day, the medium was changed and the transfected cells were allowed to secrete virus for 48-72 hours in 10 ml of medium. Culture supernatants were then filtered using 0.45 micron filters. For infection of target cells with lentivirus, the viral supernatants were diluted between 2 to 4-fold in fresh medium for transduction. Transduction was carried out in the presence of 8 µg/ml polybrene (Sigma) and a medium change was made 12-18 hours later. For selection of pLKO.1 plasmids, cells were treated with 2.5 µg/ml puromycin the day after transduction.
EGFR activation assays

HEK 293T cells were transfected in 6-well plates with the indicated nanogram amounts of untagged EGF plus 50 ng of RHBDL2. The following day, the cells were washed and then cultured in serum free medium in the presence of 10 µM BB94. 36 hours later, the culture supernatants were harvested, centrifuged to remove cell debris and then applied to a sub-confluent monolayer of A431 cells which had previously been serum starved for 24 hours. Ten minutes later, the HEK 293T supernatants were aspirated off and the stimulated A431 monolayer was lysed in 1x SDS PAGE sample buffer. Samples of the A431 lysates were immunoblotted with anti-phospho EGFR and total EGFR antibodies. In addition, samples of the original EGF-containing culture supernatants from 293T cells were also electrophoresed.

Supplemental Figure Legends

Figure S1

Failure of RHBDL2 to cleave Neuregulins. COS cells were transfected with the indicated plasmids including the rat Nrg1 isoforms NDF β4a (A), rat Glial Cell Growth factor (B) human Myc-tagged Nrg4 (C) and Myc-EGF (D) in the presence of WT or SA mutant RHBDL2. A secretion assay was performed in the absence or presence of BB94 as described in Materials and Methods and westerns were probed with anti-NDF antibodies (A,B) or anti-Myc antibodies (C,D). FLAG-prolactin served as a secretion control and RHBDL2 expression was detected with anti-HA antibodies.

Figure S2

Cleavage of EGF by RHBDL2 in HEK293ET cells in the absence of metalloprotease inhibition. HEK293ET cells were transfected with
500 ng Myc-EGF, 50 ng RHBDL2 and 25 ng FLAG-Prolactin in a total of 2 µg DNA per 6-well plate. A secretion assay was performed for 30 hours +/- 10 µM BB94 and Myc-EGF and FLAG-Prolactin was detected in culture media by western blotting.

References


Adrain_FigS1.