ADAM17 is essential for ectodomain shedding of the EGF-receptor ligand amphiregulin

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The epidermal growth factor (EGF)-receptor ligand amphiregulin (AREG) is a potent growth factor implicated in proliferative skin diseases and in primary and metastatic epithelial cancers. AREG, synthesized as a propeptide, requires conversion to an active peptide by metalloproteases by a process known as ectodomain shedding. Although (ADAM17) a disintegrin and metalloprotease 17 is a key sheddase of AREG, ADAM8-, ADAM15-, and batimastat (broad metalloprotease inhibitor)-sensitive metalloproteases have also been implicated in AREG shedding. In the present study, using a curly bare (Rhbdf2<sup>cub</sup>) mouse model that shows loss-of-hair, enlarged sebaceous gland, and rapid cutaneous wound-healing phenotypes mediated by enhanced Areg mRNA and protein levels, we sought to identify the principal ectodomain sheddase of AREG. To this end, we generated Rhbdf2<sup>cub</sup> mice lacking ADAM17 specifically in the skin and examined the above phenotypes of Rhbdf2<sup>cub</sup> mice. We find that ADAM17 deficiency in the skin of Rhbdf2<sup>cub</sup> mice restores a full hair coat, prevents sebaceous gland enlargement, and impairs the rapid wound-healing phenotype observed in Rhbdf2<sup>cub</sup> mice. Furthermore, in vitro, stimulated shedding of AREG is abolished in Rhbdf2<sup>cub</sup> mouse embryonic keratinocytes lacking ADAM17. Thus, our data support previous findings demonstrating that ADAM17 is the major ectodomain sheddase of AREG.

The epidermal growth factor receptor (EGFR) pathway plays a major role in normal development, and in multiple diseases including epithelial cancers and chronic obstructive pulmonary disease, and in liver diseases [1–5]. A critical step in regulating this pathway is ectodomain shedding of type 1 transmembrane EGFR ligands from the cell surface by membrane-anchored metalloproteases [6]. For instance, type-1 transmembrane EGFR ligands, including amphiregulin (AREG), transforming growth factor alpha (TGFα), epidermal growth factor (EGF), and heparin-binding EGF (HB-EGF), are produced as inactive propeptides. In the ectodomain shedding process, ADAMs (a disintegrin and metalloproteases) cleave propeptides to release soluble peptides, leading to activation of the EGFR signaling pathway [7,8].

Among the multiple ADAMs studied (ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, and ADAM19), ADAM10 and ADAM17 have emerged as key sheddases of the EGFR ligands EGF, betacellulin, HB-EGF, and TGFA [9,10]. In culture, ectodomain shedding assays using mouse embryonic fibroblasts (mEFs) lacking TGFA showed impaired shedding of EGF and betacellulin, and HB-EGF or TGFA, respectively [11]. In line with these findings, ADAM17 knockout mice show defects...
in cardiac valve and eyelid development [8,12], defects that are also observed in mice deficient in HB-EGF (Hbegf<sup>−/−</sup> mice) and in TGFA (Tgfa<sup>−/−</sup> mice), respectively [12–14]. Furthermore, using loss-of-function experiments in mEFs, Sahin et al. [9] demonstrated that both constitutive shedding and stimulated ectodomain shedding of EGFR ligands, including EGF, epiregulin, betacellulin, HB-EGF, TGFA, and AREG, are unaltered in the absence of ADAM8, ADAM9, ADAM12, ADAM15, and ADAM19. Thus, substantial literature suggests that ADAM10 and ADAM17 have essential, but distinct, roles in shedding of EGFR ligands.

Results of in vivo studies implicate ADAM17 as the specific metalloprotease contributing to the ectodomain shedding of AREG [15], a potent growth factor implicated in proliferative skin diseases, and primary and metastatic epithelial cancers [16–18]. Moreover, results of in vitro studies have suggested ADAM17 as a key sheddase [9,10]; nevertheless, ADAM8-, ADAM15-, and batimastat (broad metalloprotease inhibitor)-sensitive metalloproteases have also been implicated in AREG shedding in vitro [11]. Understanding of the sheddase mechanisms for AREG is critical for development of more effective therapies for diseases associated with this growth factor.

To determine whether ADAM17 is the key sheddase of AREG, we utilized the curly bare (Rhdhf2<sup>cub</sup>) gain-of-function mouse mutation. Homozygosity for this spontaneous mutation in the Rhdhf2 gene augments Areg mRNA and protein levels and results in alopecia, sebaceous gland enlargement, and rapid wound-healing phenotypes through enhanced secretion of AREG and subsequent hyperactivation of the EGFR pathway [19]. Furthermore, AREG deficiency in Rhdhf2<sup>cub/cub</sup> mice prevents the alopecia, sebaceous gland enlargement, and rapid wound-healing phenotypes, suggesting that AREG is the primary mediator of the Rhdhf2<sup>cub</sup> phenotype [19]. Thus, the Rhdhf2<sup>cub</sup> mouse mutation provides a powerful in vivo model system that allows us to examine the physiological role of ADAM17 in ectodomain shedding of AREG and in AREG-mediated downstream events, including wound healing.

Here, we demonstrate that conditional deletion of ADAM17 in the skin of Rhdhf2<sup>cub/cub</sup> mice impairs the AREG-mediated hair, sebaceous gland, and wound-healing phenotypes observed in these mice. We also demonstrate that ADAM17 deficiency significantly abolishes both stimulated and unstimulated shedding of AREG in Rhdhf2<sup>cub/cub</sup> mouse embryonic keratinocytes (MEKs), suggesting that ADAM17 is indispensable for sheddase of AREG.

Materials and methods

Animals

All animal work conformed to regulations in the Guide for the Care And Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 8th edition, 2011). Euthanasia was performed in a manner consistent with the 2013 recommendations of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia. All individuals working with animals in this project read and adhered to The Jackson Laboratory policy, POL.AWC.025 Euthanasia in Animal Experiments Involving Pain, Distress, or Illness. The Rhdhf2<sup>cub/cub</sup>, Rhdhf2<sup>−/−</sup>, and Rhdhf2<sup>cub/cub</sup> Areg<sup>−/−</sup> mice are maintained on the C57BL/6J genetic background, and Adam17<sup>flx/flx</sup> and Adam17<sup>flx/flx</sup> K14-Cre mice are of mixed genetic background [20]. We generated Rhdhf2<sup>cub/cub Adam17<sup>flx/flx</sup> K14-Cre mice by crossing female Rhdhf2<sup>cub/cub</sup>, Adam17<sup>+</sup> /flx, K14-Cre mice with male Rhdhf2<sup>cub/cub</sup> , Adam17<sup>flx/flx</sup> mice. Areg<sup>Mcub/Mcub</sup> mice are referred to as Areg<sup>−/−</sup> mice in this manuscript [19]. Mice were maintained under modified barrier conditions on a 12-h light and 12-h dark cycle with constant temperature and humidity. The Animal Care and Use Committee at The Jackson Laboratory approved all of the experimental procedures.

Histology

Mice were euthanized by CO2 asphyxiation followed by open chest necropsy, a secondary method of euthanasia. Dorsal skin was removed, fixed in 10% neutral buffered formalin for 24 h, processed routinely, embedded in paraffin, sectioned and stained, with hematoxylin and eosin (H&E).

Isolation of primary keratinocytes

For isolation of MEKs, skin from embryonic day 18 mouse embryos was incubated overnight in neutral protease at 4 °C. Following separation of the epidermis from the dermis, the epidermis was placed in Petri dishes containing trypsin (#12563029; ThermoFisher Scientific, Waltham, MA, USA) and allowed to incubate for 30 min at room temperature. After blocking trypsin activity with soybean trypsin inhibitor (#R007100; ThermoFisher Scientific), cells were grown in KBM-2 medium (#CC-3107; Lonza, Fisher Scientific, Pittsburgh, PA, USA) supplemented with antibiotic/antimycotic.

Measurement of amphiregulin protein levels

AREG levels in the cell culture supernatant were measured via ELISA as described previously [19]. Briefly, 100 μL of cell culture supernatant was added to capture antibody precoated
plates and incubated for 2 h at room temperature (RT). After three washes, 100 μL of the detection antibody was added to each well and incubated for an additional 2 h at RT. Following three washes, 100 μL of streptavidin/HRP was added to each well and incubated at RT for 20 min, before adding 100 μL of substrate solution (20 min incubation) and 50 μL of stop solution. A spectrophotometer (SpectraMax 190; Molecular Devices, San Jose, CA, USA) was used to determine the optical density.

Statistical analysis

One-way ANOVA and two-way ANOVA were used for comparison of several groups using PRISM v7 software (GraphPad, La Jolla, CA, USA). A *P < 0.05 was considered statistically significant. Data represent mean ± SD.

Results

The loss-of-hair, enlarged sebaceous gland, and rapid wound-healing phenotypes of Rhbdf2<sup>cub/cub</sup> mice are mediated through ADAM17

To determine whether ADAM17 is essential for the loss-of-hair and enlarged sebaceous gland phenotypes exhibited by Rhbdf2<sup>cub/cub</sup> mice, we generated Rhbdf2<sup>cub/cub</sup> mice lacking ADAM17 in skin, by crossing Rhbdf2<sup>cub/cub</sup> mice with Adam17<sup>flox/flox</sup> K14-Cre mice, and studied the phenotypes of second-generation offspring. We noted that ADAM17 acts as a genetic modifier of Rhbdf2<sup>cub/cub</sup> mice – Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice display a full hair coat, in contrast to the complete hair loss in Rhbdf2<sup>cub/cub</sup> mice (Fig. 1A). We next performed histopathological examination of truncal skin from Rhbdf2<sup>cub/cub</sup> (Fig. 1B.a,b) and Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre (Fig. 1B.c,d) mice at 3 weeks of age. Although, gross examination of Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice showed a full hair coat (Fig. 1A, 3), histological examination revealed mild follicular dystrophy (arrowhead) in Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice compared with extreme follicular dystrophy in Rhbdf2<sup>cub/cub</sup> mice (arrows). Additionally, mice of both strains exhibited hyperkeratosis (H) and thickened epidermis (E), whereas enlargement of sebaceous glands (*) was observed only in Rhbdf2<sup>cub/cub</sup> mice, suggesting that deletion of ADAM17 partially reverses the loss-of-hair and sebaceous gland phenotypes of Rhbdf2<sup>cub/cub</sup> mice.

We next examined the rapid wound-healing phenotype of Rhbdf2<sup>cub/cub</sup> mice. The Rhbdf2<sup>cub</sup> mutation induces a rapid wound-healing phenotype through enhanced secretion of AREG [19,21]; when we punched 2-mm through-and-through holes in the ear pinnae of Rhbdf2<sup>cub/cub</sup> mice, within 2 weeks ear-hole closure of more than 90% was observed in Rhbdf2<sup>cub/cub</sup> mice, in contrast to approximately 20% ear-hole closure in Rhbdf2<sup>+/+</sup> mice [19]. Here, we wanted to determine whether the wound-healing phenotype in Rhbdf2<sup>cub/cub</sup> mice requires ADAM17. Using the above-mentioned ear-hole closure assay, we tested the wound-healing phenotype of Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice and compared it to those of Adam17<sup>flox/flox</sup> K14-Cre and Rhbdf2<sup>cub/cub</sup> mice. Not surprisingly, impairment of wound healing was similar in Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 1C, left column) and Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 1C, middle column), whereas Rhbdf2<sup>cub/cub</sup> mice showed the rapid wound-healing phenotype (Fig. 1C, right column). Collectively, these data suggest that loss of ADAM17 in the skin of Rhbdf2<sup>cub/cub</sup> mice modifies the loss-of-hair phenotype and restores a full hair coat and diminishes the wound-healing phenotype.

Loss of ADAM17 specifically in the skin causes dermatitis and myeloproliferative disease in Rhbdf2<sup>cub/cub</sup> mice

A previous study showed that sustained deficiency of ADAM17 in the epidermis of wild-type mice results in epidermal barrier defects, and subsequently dermatitis and myeloproliferative disease; that is, a significant increase in the myeloid-cell infiltration [20]. Thus, to determine whether ADAM17 deficiency also causes dermatitis in Rhbdf2<sup>cub/cub</sup> mice, we examined the skin phenotype of Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre and Adam17<sup>flox/flox</sup> control mice. The skin of Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice displayed noticeable scaling (Fig. 2A, left), indistinguishable from the phenotype of Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 2A, right). Furthermore, histological examination of H&E sections revealed a thicker hypodermis (H) and a thinner dermis (D) in Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 2B.e), in contrast to a thinner hypodermis and a thicker dermis in both Adam17<sup>flox/flox</sup> K14-Cre (Fig. 2B.e) and Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre (Fig. 2B.e) mice. Additionally, we observed epidermal thickening (asterisk), hyperkeratosis (arrow head), and considerable infiltration of inflammatory cells, including macrophages and neutrophils (arrows), in the dermis of Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 2B.e) and Rhbdf2<sup>cub/cub</sup> Adam17<sup>flox/flox</sup> K14-Cre mice (Fig. 2B.f), in contrast to Adam17<sup>flox/flox</sup> control mice (Fig. 2B.d), which did not manifest any indication of skin disease.
Next, using flow cytometry analyses we determined whether there was any indication of myeloproliferative disease in Rhbd2\textsuperscript{cub/cub} Adam17\textsuperscript{flox/flox} K14-Cre mice by quantifying the differences in the percentages of splenic macrophages (Fig. 2C, top panel) and neutrophils (Fig. 2C, bottom panel) between Rhbd2\textsuperscript{cub/cub} Adam17\textsuperscript{flox/flox} K14-Cre and Rhbd2\textsuperscript{cub/cub} ADAM17\textsuperscript{flox/flox} K14-Cre mice. Compared with control mice (Adam17\textsuperscript{flox/flox} ADAM17\textsuperscript{flox/+} mice), we observed significantly higher percentages of macrophages and neutrophils in both of ADAM17\textsuperscript{flox/flox} K14-Cre and Rhbd2\textsuperscript{cub/cub} Adam17\textsuperscript{flox/flox} K14-Cre mice, suggesting that loss of ADAM17 specifically in the skin results in considerable myeloproliferation in Rhbd2\textsuperscript{cub/cub} mice. Taken together, our results indicate that lack of ADAM17 in the skin results in dermatitis and myeloproliferative disease, which validates previous findings by Franzke \textit{et al.} [20] that ADAM17 maintains the skin barrier. Moreover, our results showing development of a similar overt skin phenotype observed by Franzke \textit{et al.} and restoration of hair growth in Rhbd2\textsuperscript{cub/cub} mice lacking ADAM17 implicate ADAM17 as a participant in ectodomain shedding of AREG.

**Ectodomain shedding of AREG requires ADAM17**

To determine whether ADAM17 is required for AREG ectodomain shedding, we asked whether loss of ADAM17 in Rhbd2\textsuperscript{cub/cub} keratinocytes alters AREG
secretion with or without stimulation with 100 nM phorbol-12-myristate-13-acetate (PMA). We also examined AREG-stimulated and AREG-unstimulated secretion in Rhbd2+/+ and Rhbd2cub/cub ADAM17+/+ control mice (1) and Rhbd2+/+ ADAM17flox/flox K14-Cre (2) mice. First, in line with our previous findings [19], we found that both the stimulated secretion (red column) and unstimulated (blue column) secretion of AREG are significantly increased in Rhbd2cub/cub keratinocytes compared with control keratinocytes (Fig. 3A; 1, Rhbd2+/+; 2, Rhbd2+/+/K14-Cre). Note the relatively thicker dermis (D) and thinner hypodermis (H) in both Rhbd2+/+ ADAM17flox/flox K14-Cre (b) and Rhbd2cub/cub ADAM17flox/flox K14-Cre (c) mice, compared to the thinner dermis and thicker hypodermis in Rhbd2+/+ ADAM17flox/flox K14-Cre control mice (a). Scale bars: 250 μm (low magnification) and 50 μm (high magnification). (C) Both ADAM17flox/flox K14-Cre and Rhbd2cub/cub ADAM17flox/flox K14-Cre mice develop myeloproliferative disease, evidenced by the increased percentage of macrophages (top) and neutrophils (bottom) in the spleens of these mice compared with control mice (ADAM17flox/flox and ADAM17flox/+ mice).

Fig. 2. (A) The role of ADAM17 in regulating the skin barrier has been established previously [20] – deletion of ADAM17 in the skin results in epidermal defects and dermatitis. A similar phenotype – dry scaly skin (arrows) – was observed in both Adam17flox/flox K14-Cre (1) and Rhbd2cub/cub ADAM17flox/flox K14-Cre (2) mice. (B) Deletion of ADAM17 in the skin results in epidermal defects and dermatitis. A similar dermatitis-like phenotype, including epidermal thickening (asterisk), hyperkeratosis (arrow head), and considerable infiltration of inflammatory cells (arrows), was observed in both Adam17flox/flox K14-Cre (b, e) and Rhbd2cub/cub ADAM17flox/flox K14-Cre (c, f) mice, compared to the normal skin in Adam17flox/flox control mice (a, d). Note the relatively thicker dermis (D) and thinner hypodermis (H) in both Adam17flox/flox K14-Cre (b) and Rhbd2cub/cub ADAM17flox/flox K14-Cre (c) mice, compared to the thinner dermis and thicker hypodermis in Adam17flox/flox control mice (a). Scale bars: 250 μm (low magnification) and 50 μm (high magnification). (C) Both ADAM17flox/flox K14-Cre and Rhbd2cub/cub ADAM17flox/flox K14-Cre mice develop myeloproliferative disease, evidenced by the increased percentage of macrophages (top) and neutrophils (bottom) in the spleens of these mice compared with control mice (ADAM17flox/flox and ADAM17flox/+ mice).
hair growth and loss of rapid wound-healing phenotypes in $\text{Rhbdf}2^{\text{cub/cub}} \text{Adam17}^{\text{flox/flox}} \text{K14-Cre}$ mice, we observed that $\text{Rhbdf}2^{\text{cub/cub}} \text{Adam17}^{\text{flox/flox}} \text{K14-Cre}$ keratinocytes failed to secrete AREG even after stimulation with PMA (Fig. 3A; 6), in contrast to a significant increase in both stimulated and unstimulated $\text{Rhbdf}2^{\text{cub/cub}}$ keratinocytes (Fig. 3A; 2), suggesting that the $\text{Rhbdf}2^{\text{cub}}$ mutation fails to promote AREG secretion in the absence of ADAM17. Notably, the levels of AREG observed in keratinocytes lacking ADAM17 (Fig. 3A; 5 and 6) can be attributed to constitutive shedding, which does not require ectodomain shedding by metalloproteases [9].

Fourth, similar results were obtained when keratinocytes isolated from the aforementioned strains (1 through 6) of mice were exposed to bacterial endotoxin lipopolysaccharide (LPS) and assayed for AREG levels in the culture supernatants (Fig. 3B). Interestingly, there was a subtle but significant increase in the levels of AREG in $\text{Adam17}^{\text{flox/flox}} \text{K14-Cre}$ and $\text{Rhbdf}2^{\text{cub/cub}} \text{Adam17}^{\text{flox/flox}} \text{K14-Cre}$ keratinocytes upon stimulation with LPS (Fig. 3B; 5 and 6); however, this could be due to differential regulation of AREG constitutive shedding by PMA versus LPS. Taken together, because loss of ADAM17 significantly abolished stimulated secretion of AREG in $\text{Rhbdf}2^{\text{cub/cub}}$ keratinocytes, these data strongly suggest that ADAM17 is essential for ectodomain shedding of AREG (Fig. 3C).

**Discussion**

Amphiregulin plays an important role in pathological processes, including psoriasis induction [22,23], cancer progression, and resistance to chemotherapy and anti-EGFR therapies [16,24]. For example, AREG has been characterized as a multocrine – autocrine, paracrine, and endocrine (systemic) – growth factor in primary and metastatic epithelial cancers [25–27]. AREG induces its own expression to enable self-sufficiency of growth signals acting through EGFR, via an extracellular autocrine loop [28], suggesting that dysregulation of this loop could lead to overexpression of AREG. Additionally, cancer cells overexpressing AREG can induce neoplastic transformation of neighboring cells through paracrine or endocrine activity [15]. Also, more recently, we showed in mice that AREG underlies the hyperproliferative skin disease tylosis and that
loss of AREG restores the normal skin phenotype in a mouse model of human tylosis [29]. Together, these studies highlight the key role of AREG in several pathological processes, and the potential of AREG depletion as a therapeutic approach in multiple diseases. To develop effective therapeutic strategies targeting AREG, it is important to understand how AREG secretion is regulated in vivo.

Amphiregulin synthesized as pro-AREG is converted to an active form by metalloproteases. Although several ADAMs have been implicated, a study by Sahin et al. [9] showed that $Adam17^{+/−}$ MEFs exhibit impaired shedding of AREG, indicating that ADAM17 may be a major sheddase. Comparison of the phenotype of $Adam17^{+/−}$ mice with that of $Arg^{-/−}$ mice is a potential means of providing support for a role of ADAM17 as the major sheddase of AREG. However, literature suggests that in contrast to $Hbegf^{−/−}$ and $Tgα^{−/−}$ mice [13,14], $Arg^{-/−}$ mice are viable and do not present with an overt phenotype, except for defects in mammary gland development during puberty and in nursing [30]. Thus, it remains to be determined whether the phenotype of mice with $Arg$ depletion resembles any aspects of the $Adam17^{+/−}$ phenotype. $Adam17^{−/−}$ mice exhibit perinatal lethality, limiting the ability to examine mammary gland development and nursing competence phenotypes. Furthermore, although at birth $Adam17^{−/−}$ pups exhibit stunted growth and development [31], including defective mammary branching, suggesting a role for ADAM17 in shedding of AREG, there is a lack of direct evidence. In the present study, using mouse genetics and in vitro ectodomain shedding assays, we sought to determine whether loss of ADAM17 abolishes shedding of AREG in vivo. We demonstrate that loss of ADAM17 impairs the AREG-mediated loss-of-hair, enlarged sebaceous gland, and rapid wound-healing phenotypes observed in $Rhbdf2^{−/−}$ mice. Moreover, we find that conditional deletion of ADAM17 in the skin of $Rhbdf2^{−/−}$ mice significantly inhibits stimulated secretion of AREG in keratinocytes, suggesting that ADAM17 is necessary for ectodomain shedding of AREG in keratinocytes. Notably, in macrophages, RHBDF2 also regulates stimulated secretion of pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) through ADAM17 [32,33], implicating that, similar to ectodomain shedding of AREG, ADAM17 might be key for shedding of RHBDF2-mediated secretion of TNFα. Consistently, RHBDF2 has recently been suggested to be an essential regulator of stimulated growth factor and cytokine signaling via ADAM17 [34,35].

We previously observed that $Rhbdf2$ gain-of-function alleles, including $Rhbdf2^{−/−}$, could induce secretion of AREG in the presence of a potent ADAM17 inhibitor marimastat [19]. Moreover, both our group [19] and Siggs et al. [36] observed that ADAM17 activity is reduced in $Rhbdf2^{−/−}$ mice. Based on these findings, we postulated that RHBDF2 might regulate secretion of AREG independent of ADAM17 activity [19]. However, results from the present study showing that loss of ADAM17 reverses the phenotype of $Rhbdf2^{−/−}$ mice suggest that ADAM17 is indispensable for sheddase of AREG and that RHBDF2 does not act as a sheddase of AREG.

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Author contributions

VH, LDS, and MVW designed research; VH, LMB, and MLF performed experiments; VH, LMB, and MLF acquired data; VH, LDS, and MVW analyzed data; and VH, LDS, and MVW wrote the paper.

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