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Epidermal ADAM17 is dispensable for Notch activation

Arjan J. Groot[2,#], **Cristina Cobzaru**[1,#], **Silvio Weber**[3], **Paul Saftig**[4], **Carl P. Blobel**[5], **Raphael Kopan**[6], **Marc Vooijs**[2,¶], and **Claus-Werner Franzke**[1,¶]

[1]Dept. of Dermatology, University Freiburg - Medical Center, Germany [2]Dept. of Radiotherapy (MAASTRO)/GROW - School for Oncology & Developmental Biology, University of Maastricht, Maastricht, The Netherlands [3]Heart Research Centre Göttingen, Universitätsmedizin Göttingen, Dept. of Cardiology and Pneumology, University Göttingen, Germany [4]Institute of Biochemistry, University Kiel, Germany [5]Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY, USA [6]Washington University School of Medicine, St. Louis, MO, USA

To the Editor:

Proteases of the ADAM (A disintegrin and metalloproteinase) family form a large group of metalloenzymes that function primarily to cleave (or shed) extracellular domains from plasma membrane bound signaling molecules. Whereas ADAM proteases are not thought to have strict cleavage site specificities, ADAM17 and ADAM10 have evolved distinct substrate preferences despite being the most closely related members of the family. ADAM17 is the principal sheddase of TNF-α and the majority of the EGFR-ligands (Blobel, 2005; Peschon *et al.*, 1998), whereas ADAM10 is required for Notch and amyloid precursor protein (APP) shedding (Kuhn *et al.*, 2010; van Tetering *et al.*, 2009). The physiological importance of ADAM17 in EGFR-signaling is based on the striking phenotypic similarities between *Adam17*$^{-/-}$ and *Egfr*$^{-/-}$ mutant mice. Both die at birth due to multiple developmental defects involving the heart, lung, and skin (Blobel, 2005; Miettinen *et al.*, 1995; Peschon *et al.*, 1998).

Notch receptors are conserved membrane-anchored transcription regulators activated by proteolysis (Kopan and Ilagan, 2009; van Tetering and Vooijs, 2011). During maturation in the Golgi, Notch receptors are cleaved by furin-like convertases at site 1 (S1) located in a loop protruding from the negative regulatory region (NRR). When denatured for western blot analysis, the membrane–bound form of Notch1 (TMIC) migrates at ~120 kDa. Ligand binding unfolds the Lin12-Notch repeat (LNR) module to expose and allow cleavage at

Correspondence: Claus-Werner Franzke, Dept. of Dermatology, University Freiburg - Medical Center, Hauptstrasse 7, 79104 Freiburg, Germany, Tel: +49 761 27067850, Fax: +49 761 27067200; claus-werner.franzke@uniklinik-freiburg.de**OR** Marc Vooijs, Dept of Radiotherapy (MAASTRO)/GROW – School for Oncology and Developmental Biology, Maastricht University, Universiteitssingel 50, 6229 ER Maastricht, The Netherlands, Tel: + 31 (0)43 388 2912; Fax: + 31 (0)43 388 4540; marc.vooijs@maastrichtuniversity.nl.

[#]equal contribution

[¶]equal contribution

Conflict of interest

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Val1711 (“S2”) creating “NEXT”, which is 55 amino acids shorter than TMIC. S2 cleavage is followed by γ-secretase cleavage at Val1744 (“S3”), releasing the ~110 kDa Notch intracellular domain (NICD) that acts as a transcriptional regulator (Figure S1). In all metazoan, the essential role of ADAM10 and its orthologs (Kuzbanian and Sup-17) in executing the Notch S2 cleavage is evident by the phenotypic similarities between *Notch* and *Adam10/Kuz/Sup-17* mutants in mice, flies and worms, respectively (van Tetering and Vooijs, 2011).

ADAM17 can mediate S2 cleavage under special, non-physiological circumstances. It cleaves T-ALL-associated, mutant forms of Notch that are ligand independent in tissue culture cells (Bozkulak and Weinmaster, 2009). It also cleaves Notch proteins unfolded *in vitro* with Ca^{2+} chelators (Rand *et al.*, 2000); however, a physiological activity for ADAM17 in S2 cleavage should be ruled out for two reasons. First, most *Adam10* loss of function phenotypes examined to date (including skin phenotypes) phenocopied *Notch* loss (Demehri *et al.*, 2008; Dumortier *et al.*, 2010; Weber *et al.*, 2011) indicating that ADAM17 cannot rescue S2 cleavage in *Adam10* deficient tissues. Second, in cell-based assays, ligand-induced Notch signaling depends on ADAM10 (Bozkulak and Weinmaster, 2009; van Tetering *et al.*, 2009) and occurs at Val1711 S2 cleavage site during ligand dependent Notch1 signaling (van Tetering *et al.*, 2009).

Recently, Murthy and colleagues (Murthy *et al.*, 2012) suggested a role for ADAM17 in maintaining a baseline level of Notch1 activity (“tonic” Notch signal) in epidermal keratinocytes in a ligand-independent manner. We examined the data supporting this function in the same genetic model used by Murthy et al, arriving at different conclusions. Both Murthy et al. (Murthy *et al.*, 2012) and Franzke et al. (Franzke *et al.*, 2012) characterized the same keratinocyte-specific *Adam17* knockout mouse model (*Krt14-A17*$^{\Delta KC}$), and observed altered epidermal differentiation and the onset of chronic dermatitis and severe myeloproliferative disease. Although both studies concur that ADAM10 is required for ligand-dependent Notch signaling during skin development, Murthy et al., concluded that *Adam17* loss alleviated a Notch-dependent suppression of pro-inflammatory cytokine thymic stromal lymphopoietin (TSLP). According to Murthy and colleagues, ADAM17 maintains a ligand-independent (“tonic”) mode of Notch signaling in skin, which in turn suppressed TSLP production. Measurements of TSLP by ELISA demonstrate that γ-secretase-deficient keratinocytes produced indistinguishable amounts of TSLP from the wild type cells (Demehri *et al.*, 2008) however are inconsistent with the idea that a “tonic” Notch signal regulates TSLP in skin.

We have provided an alternative explanation by showing that loss of ADAM17-dependent EGFR signaling impaired the maintenance of epidermal differentiation and barrier integrity in *Krt14-A17*$^{\Delta KC}$ mice (Franzke *et al.*, 2012), which would be sufficient for TSLP induction as demonstrated in several animal models with epidermal barrier defects, including *Gata3*$^{-/-}$ (de Guzman Strong *et al.*, 2006), epidermis-specific loss of *Notch* (Demehri *et al.*, 2008), or mice lacking the lipid transporter *FATP4* (Demehri et al., 2008). Mice with a persistent defect in epidermal barrier maintenance have chronically elevated TSLP and GCSF expression, which together with additional cytokines drive leukocyte infiltration and promote development of atopic dermatitis, myeloproliferative disease, and asthma. Because

this TSLP production also occurs in mice with a competent Notch signaling system but a defective epidermal differentiation program, TSLP expression is triggered by a mechanism that does not involve a cell autonomous Notch function (Demehri *et al.*, 2008).

In addition to the genetic and cell-based data summarized above, both themethods and the interpretation of the data, which supported a physiological role for ADAM17 in tonic Notch signaling, raise several concerns. To support the conclusion that ADAM17 regulates ligand-independent Notch signaling, Murthy and colleagues activated Notch with EDTA, an *in vitro* assay with no physiological correlate rendering Notch permissive to cleavage by either ADAM10 or ADAM17. To provide physiological evidence, they presented data suggesting that NICD amounts were reduced in *Krt14-A17*$^{\Delta KC}$ epidermis *in vivo* at postnatal day 56 (P56) *versus* control mice. A 72 kDa band labeled as NICD (Val1744) is greatly diminished in *Adam17*-deficient epidermis (Murthy *et al.*, 2012). Based on the molecular weight of an intact NICD (~110kDa in SDS PAGE), this fragment is most likely a metabolic product of NICD that retained the VLLS epitope but lost a fraction of its C-terminus. Since ADAM10 is present, it was unclear to us why loss of “tonic” signal will cause such a pronounced change in NICD levels.

To evaluate the contribution of ADAM17 to NICD production *in vivo*, we repeated this experiment using epidermal lysates from the same *Krt14-17*$^{\Delta KC}$ mice (named *Adam17*$^{\Delta ep}$ in (Murthy *et al.*, 2012)) and analyzed these at the same and more stages for Notch proteolysis. Due to the small differences in molecular weight between NEXT and NICD (33 amino acid residues), we visualized NICD with cleavage-specific antibodies (Figure 1A). We analyzed the epidermis from newborn, P10 and P56 *Krt14-A17*$^{\Delta KC}$ epidermis using an antibody recognizing the C-terminus to identify total Notch1 (TMIC) and a Val1744 neo-epitope antibody to detect NICD unequivocally by western blot. As expected, NICD production in *Krt14-A17*$^{\Delta KC}$, *Krt14-Egfr*$^{\Delta KC}$ and control epidermis is indistinguishable in all postnatal ages, consistent with an intact, ADAM10-dependent, canonical signaling pathway. In contrast NICD production in the *Krt14-A10*$^{\Delta KC}$ (named *Adam10*$^{epi-/-}$ in (Weber *et al.*, 2011)) epidermis at P1 was impaired indicating ADAM10 is the sheddase of Notch1 in the epidermis (Figure 1A) (Weber *et al.*, 2011).

If “tonic” Notch signaling exists, loss of tonic signals predicts that elevated TSLP production (released from Notch mediated inhibition) should precede (perhaps even drive) the defects in epidermal differentiation and barrier integrity, as seen in *Notch* and *Fatp4* deficient animals (Demehri *et al.*, 2008). Conversely, if EGFR deficiency caused barrier defects that then trigger *Tslp* expression, TSLP levels should rise only after the barrier defects are manifested. In order to analyze the time course of keratinocyte derived TSLP production in *Krt14-A17*$^{\Delta KC}$ animals, we measured the *Tslp* mRNA in P10, P19, and P56 skin. Importantly, *Tslp* mRNA expression in P10 skin is identical to controls. Only when pronounced differentiation and barrier defects were noted (P19) (Franzke *et al.*, 2012) did *Tslp* mRNA accumulate, and stayed consistently elevated in the skin of two-month-old animals (Figure 1B). Accordingly, TSLP serum levels were significantly increased in P19 and P56 animals, but unchanged compared to controls at P10 preceding barrier dysfunction (Figure 1C). In addition, we have seen the same time course for an increase in TSLP in *Krt14-Egfr*$^{\Delta KC}$ mice (Figure 1B, C). These results argue against a cell intrinsic upregulation

of keratinocyte *Tslp* through loss of Notch-dependent suppression and support the null hypothesis that TSLP production follows barrier disruption (Demehri *et al.*, 2008).

Since *Adam17* disruption in these studies is achieved using the identical *Krt14-A17*$^{\Delta KC}$ animal model, and since deletion of *Adam17* in keratinocytes did not lead to elevation of *Tslp* mRNA until after a barrier defect was detected, *Adam17* loss was unlikely to be associated with reduced epidermal Notch signaling *in vivo*. All the data can be explained by strongly diminished activation of EGFR in the epidermis of *Krt14-A17*$^{\Delta KC}$ mice caused by reduced ADAM17 dependent EGFR-ligand shedding, which led to reduced expression of transglutaminases, reducing crosslinking of barrier proteins and thereby causing a differentiation and skin barrier defect (Franzke *et al.*, 2012). Though it is possible that ADAM17 may cleave Notch proteins *in vivo* under some yet to be described circumstances, the data and analysis provided herein do not support the “tonic” Notch signaling inhibiting TSLP hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
EGFR	epidermal growth factor receptor
NICD	Notch intracellular domain
TSLP	thymic stromal lymphopoietin

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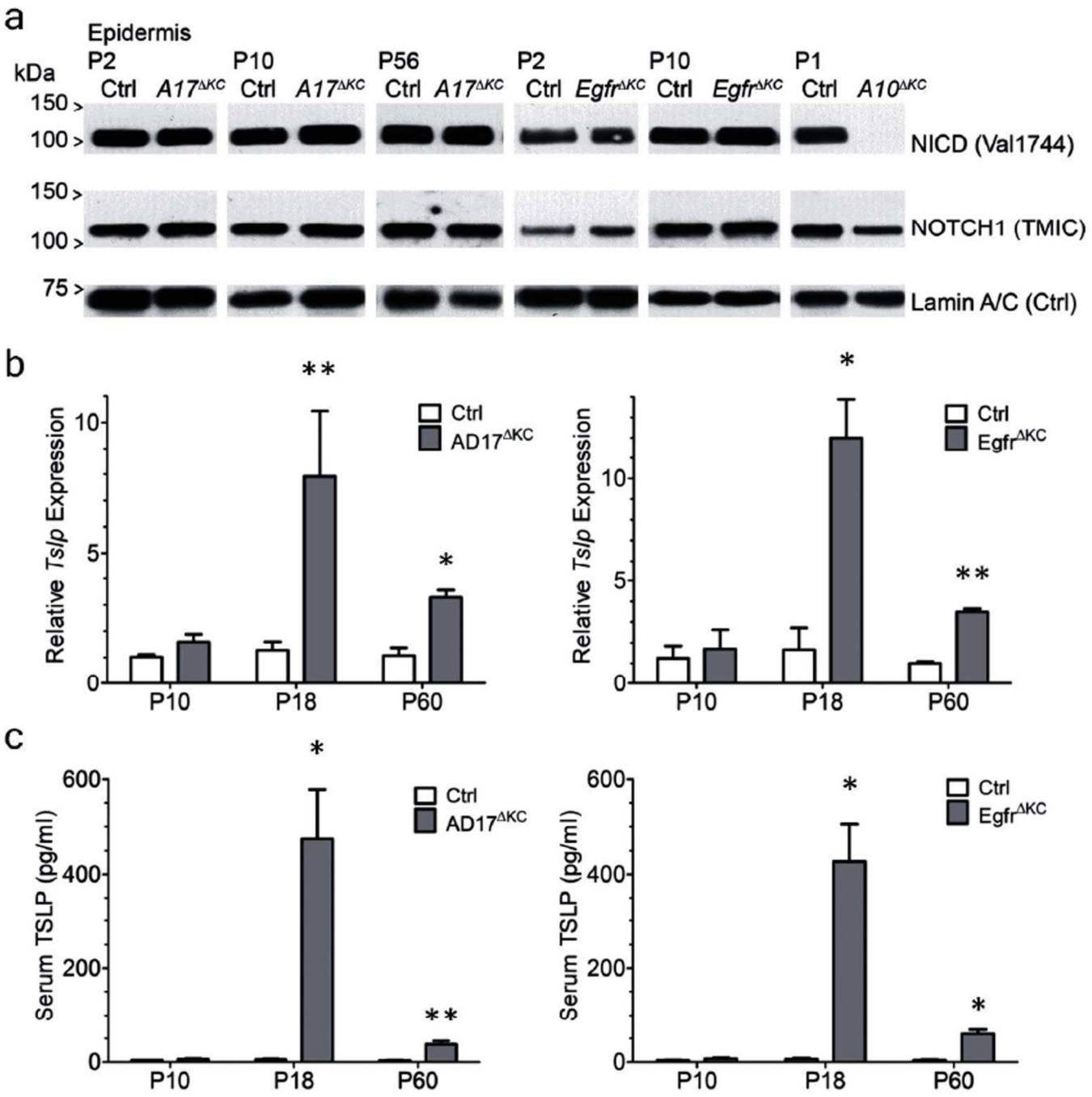

Figure 1. Keratinocyte-derived ADAM17 is dispensable for Notch1 activation and the upregulation of the epidermal alarmin TSLP in *Krt14-A17*$^{\Delta KC}$ mice depends on skin barrier defects

(A) Western blot analysis of epidermal lysates for activated S3 cleaved NOTCH1; NICD (Val1744, upper panels) in *Krt14-A17*$^{\Delta KC}$ and *Krt14-Egfr*$^{\Delta KC}$ mice revealed no differences to their littermate controls at different postnatal ages, while NICD was completely lost in the epidermis of *Krt14-A10*$^{\Delta KC}$ animals. NOTCH1 protein (TMIC, middle panel) and Lamin A/C (Ctrl, bottom panels) were used as loading controls. Data from each postnatal age are representative of at least three mice (for each genotype). (B) Quantitative *Tslp* gene

expression analysis of *Krt14-A17*$^{\Delta KC}$ or *Krt14-Egfr*$^{\Delta KC}$ skin revealed no changes in expression on P10, but significantly elevated expression at P19 and P56, when the skin barrier defects are prominent. Mice analyzed for *Krt14-A17*^{KC} and controls: P10, n=5; P19, n=5; P56, n=4; and for *Krt14-Egfr*$^{\Delta KC}$ and control littermates n=3 for all postnatal ages. (C) Analysis of serum TSLP levels of *Krt14-A17*$^{\Delta KC}$ or *Krt14-Egfr*$^{\Delta KC}$ animals at P10, P19, and P56 revealed increased TSLP levels due to severe skin barrier defects starting at P19. The data derived from three different mice for each genotype. Data in B and C are shown as mean ± SEM, *p<0.05; **p<0.01.