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Running title: EDAR in adult sebaceous glands.

Abbreviations: EDA: Ectodysplasin A. EDAR: EDA receptor. HED: hypohidrotic ectodermal dysplasia. XLHED: X-linked HED. TAG: triacylglycerol.

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Abstract

Impaired Ectodysplasin A (EDA) – EDA receptor (EDAR) signaling affects ectodermally derived structures including teeth, hair follicles and cutaneous glands. X-linked hypohidrotic ectodermal dysplasia (XLHED), resulting from *EDA* deficiency, can be rescued with lifelong benefits in animal models by stimulation of ectodermal appendage development with EDAR agonists. Treatments initiated later in the developmental period restore progressively fewer of the affected structures. It is unknown whether EDAR stimulation in adults with XLHED might have beneficial effects. In adult *Eda* mutant mice treated for several weeks with agonist anti-EDAR antibodies, we find that sebaceous glands size and function can be restored to wild type levels. This effect is maintained upon chronic treatment but reverses slowly upon cessation of treatment. Sebaceous glands in all skin regions respond to treatment, though to varying degrees, and this is accompanied in both *Eda* mutant and wild type mice by sebum secretion to levels higher than those observed in untreated controls. *Edar* is expressed at the periphery of the glands, suggesting a direct homeostatic effect of Edar stimulation on the sebaceous gland. Sebaceous gland size and sebum production may serve as biomarkers for EDAR stimulation, and EDAR agonists may improve skin dryness and eczema frequently observed in XLHED.

Introduction

Hypohidrotic ectodermal dysplasia (HED) is a congenital condition defined by reduced or absent development of teeth, hair follicles and cutaneous glands, notably the eccrine sweat glands (Clarke, 1987; Clarke et al., 1987; Wright et al., 1993). This condition is caused by defects in signaling from the transmembrane receptor EDAR, most commonly due to mutation of the gene encoding its ligand, EDA, which lies on the X-chromosome (Kere et al., 1996), or caused by mutation of the *EDAR* gene itself (Monreal et al., 1999) or affecting its intracellular adapter protein EDARADD (Headon et al., 2001). Absence or reduced function of this TNF-like pathway leads to failure to activate NF-κB,

which is required for initiation of appendage development and control of morphogenesis (Doffinger et al., 2001; Schmidt-Ullrich et al., 2001).

Most individuals with HED are males with the X-linked form (XLHED), caused by mutation of the *EDA* gene. Although few systematic reports of HED incidence exist, a recent Danish study estimated the frequency of XLHED at between 1.6 and 21.9 per 100,000 population, depending on the stringency of diagnostic criteria applied (Nguyen-Nielsen et al., 2013). Much of the ongoing management of XLHED-affected patients centers on ameliorating the symptoms of glandular dysfunction in the skin and mucosae. This management includes provision of lubrication to the eyes to compensate for reduction of Meibomian and lacrimal gland secretions (Dietz et al., 2013; Reed et al., 1970; Tyagi et al., 2011), administering artificial saliva due to xerostomia arising from salivary gland reduction (Daniel et al., 2002), removal of nasal and otic crusting (Callea et al., 2013) and application of emollient to improve dry skin and eczema symptoms (Chen, 2006; Tyagi et al., 2011). External cooling during physical exertion or in hot weather may also be needed due to reduced or absent sweating (Hammersen et al., 2011; Schneider et al., 2011).

Two types of pharmacological modulators of EDAR signaling are currently available. The first consists of a modified form of EDA containing its C-terminal TNF domain fused to an immunoglobulin Fc domain (Gaide and Schneider, 2003) and the second a set of monoclonal antibodies that bind the EDAR extracellular domain to stimulate this pathway (Kowalczyk et al., 2011), presumably by causing clustering and activation of the receptor. Suppression of the EDA signal may be achieved by blocking monoclonal antibodies directed against the ligand's receptor binding domain (Kowalczyk-Quintas et al., 2014).

In the mouse, mutation of any of the *Eda*, *Edar* or *Edaradd* genes leads to a phenotype analogous to the human condition, with defective development of teeth, glands and certain types of hair follicles

(Headon et al., 2001; Headon and Overbeek, 1999; Srivastava et al., 1997). The morphological defects caused by *Eda* mutation can be rescued by prenatal or perinatal administration of recombinant Fc-EDA protein (Casal et al., 2007; Gaide and Schneider, 2003), or ligand replacement using an EDAR agonist antibody (Kowalczyk et al., 2011), as demonstrated in mouse and dog models of XLHED. These therapeutic effects have lifelong benefit, but are achieved only if ligand is administered during a developmental window appropriate for a particular structure. Treatment after the developmental window had no detectable effect on the parameters examined. For example, shape of the first molar is rescued only if Fc-EDA is administered to mice before embryonic day 15 (Gaide and Schneider, 2003). Unknown, however, is whether dynamic structures which undergo continual cell proliferation might benefit from chronic EDAR stimulation in adult life.

The sebaceous glands undergo constant cellular turnover throughout life, driven by proliferation of the flattened cells at the gland's periphery. The daughter cells thus produced move to the center of the gland, swelling as they accumulate and modify lipids until cellular rupture and release of the sebum into the hair canal or onto the skin surface (Niemann and Horsley, 2012). The sebum itself is a complex lipid mixture composed primarily of triglycerides, cholesterol and wax esters which acts to aid skin barrier function and humidification (Fluhr et al., 2008; Fluhr et al., 2003), modulate the skin microflora (Fischer et al., 2013), deliver antioxidants to the skin surface (Thiele et al., 1999) and maintain the hair follicles (Stenn, 2001; Sundberg et al., 2000; Zheng et al., 1999). Altered sebaceous gland activity is implicated in the pathogenesis of a number of dermatological conditions, including acne vulgaris (Zouboulis et al., 2013), some forms of dermatitis (Gary, 2013) and rosacea (Raghallaigh et al., 2012), while reduced sebaceous function may be causative in hidradenitis suppurativa (Kamp et al., 2011) and scarring alopecia (Al-Zaid et al., 2011).

A characteristic of XLHED is that the sebaceous glands are sparse and hypoplastic, this likely being the underlying cause of the dry skin and susceptibility to eczema in this condition (Daniel et al.,

4

2002; Reed et al., 1970). In the *Eda^Ta* (Tabby mutant) mouse model of XLHED the sebaceous glands are reduced (Gruneberg, 1971), while constitutive expression of an *Eda* transgene causes an increase in sebaceous gland size (Cui et al., 2003; Mustonen et al., 2003). Cui et al. (Cui et al., 2003) have shown that persistent expression of *Eda* throughout development and postnatal life leads to sebaceous gland hyperplasia, but that subsequent suppression of *Eda* expression in the adult is followed by sebaceous gland reduction over a period of months. This demonstrates an impact of Eda signaling on mature sebaceous gland function, though whether this impact can be achieved when the Edar pathway is activated for the first time in the adult, never having been active during development, is unknown.

The generation of reagents that allow time-limited modulation of EDAR activity at any stage of life (Gaide and Schneider, 2003; Kowalczyk et al., 2011; Kowalczyk-Quintas et al., 2014), and the onset of clinical trials to assess the potential of these medicines to treat XLHED and some forms of autosomal HED, have prompted us to assess the responses of adult sebaceous glands to activation of this pathway. We find corrective effects at morphological and functional levels upon Edar stimulation in adult XLHED animals, demonstrating that at least some adult tissues retain an ability to respond to chronic EDAR stimulation with potential therapeutic benefit and also serving as a biomarker of EDAR stimulation *in vivo*.

Results

Rescue of sebaceous gland size in adult mice by treatment with Edar-agonist antibody

We began by assessing whether sebaceous glands respond to administration of the EDAR agonist monoclonal antibody mAbEDAR1 (Kowalczyk et al., 2011). *Eda^Ta* mice received intraperitoneal administrations of 2 mg/kg mAbEDAR1 starting at postnatal day 12 (P12), a time point when no developmental rescue of hair, sweat glands and teeth can be obtained (Gaide and Schneider, 2003).

5

Treatments were performed weekly for 24 weeks and tissue samples collected at 26 weeks of age, together with skin from untreated *Eda^Ta*, from wild type mice, and from treated *Eda^Ta* mice in which treatment was terminated 6 weeks prior to analysis to assess whether any effect obtained would be reversible in this time period (Figure 1a). Sebaceous gland size was determined in sections of facial skin (Figure 1b) and measured to determine aggregate gland size relative to skin length (Figure 1c). Untreated *Eda^Ta* mutants had hypoplastic sebaceous glands, in aggregate more than 2-fold smaller than those of wild type animals, while systemic treatment of this mutant line with mAbEDAR1 completely rescued gland size. This rescue persisted for at least one month after cessation of antibody administration. We found that sebocyte size was unchanged across treatments and genotypes (Figure 1d), indicating that reduced gland size in *Eda^Ta* skin is due to a lack of sebocytes, rather than these cells being smaller than normal. Rescue of sebaceous gland size by mAbEDAR1 administration was thus achieved by increasing cell number to that of wild type mice.

Dynamics of sebaceous gland response to Edar stimulation

In order to obtain a dynamic view of sebaceous gland responses to mAbEDAR1 administration and withdrawal, we initiated treatment of *Eda^Ta* mutant and wild type animals at weaning (between 21 and 26 days of age, *i.e.* later than P12, and in the adult hair cycle, to further exclude any contribution of developmental processes) with 2 mg/kg mAbEDAR1 and then either re-administered every second week until tissue collection or else maintained animals with no further administration (Figure 2a). Untreated animals were maintained as controls. We devised a more rapid processing method to visualize sebaceous glands in ear skin: collagenase was used to remove dermal tissue, followed by staining for lipid using Oil Red-O, a process that reveals sebaceous glands as paired kidney-shaped structures around the hair follicles (Figure 2b).

The average sebaceous gland size in ear skin was smaller in *Eda^Ta* than wild type animals across the experimental time course from 2 to 24 weeks post-weaning (Figure 2c). Gland sizes were relatively

stable at different ages, with a trend to reduced size in older individuals. Treatment with mAbEDAR1 produced no detectable effect on either Eda^{Ta} or wild type sebaceous glands after two weeks, but by six weeks an enlargement was detectable in Eda^{Ta} skin (Figure 2d). With twelve weeks of continual treatment, involving 6 administrations in total, the Eda^{Ta} glands had attained the size observed in wild type, while treated wild type animals displayed only a non-significant increase in gland size (Figure 2e). Similar effects were observed in ear skin of Eda^{Ta} mice treated for 6 months with mAbEDAR1 (Figure 1a and data not shown). The effects of a single administration to Eda^{Ta} were long-lived, being detectable at the morphological level 12 weeks later, but lost by 24 weeks (Figure 2d). Thus, despite this long-lived effect, maintenance of sebaceous rescue requires chronic treatment (Figure 2d).

Sebaceous glands in dorsal skin are highly responsive to Edar stimulation

We next analyzed sebaceous glands in the more intensively studied dorsal skin at the 12 and 24 weeks time points. Aggregate sebaceous gland size in Eda^{Ta} skin was about half that of wild type, but chronic treatment with mAbEDAR1 stimulated a large increase in the size of both mutant and wild type glands (Figure 3a,b). Similar to what was observed in the ear, a single administration of mAbEDAR1 was less efficient than chronic treatment in increasing sebaceous gland size 12 or 24 weeks later (Figure 3b). The response in dorsal skin appears less limited than in the ear skin as at the 12 weeks time point we measured 3.1-fold and 6.9-fold increases for dorsal skin sebaceous glands of wild type and Eda^{Ta} mice (Figure 3b), compared to 1.4-fold and 2.3-fold increases in ear skin sections (Figure 3c).

***Edar* expression in mature sebaceous glands**

To identify the likely cellular target stimulated by mAbEDAR1, we assessed which cell types in adult dorsal skin express the *Edar* gene. In situ hybridization of adult mouse skin in anagen phase revealed *Edar* expression at the periphery of the sebaceous glands, but occasionally also on sebocytes in wild

7

type animals but not in *Edar*-deleted (*Edar*^OVE1B/OVE1B^) mice (Headon and Overbeek, 1999) (Figure 4a), suggesting that antibody driven sebaceous gland enlargement is achieved by direct stimulation of the glands.

EDAR stimulation induces proliferation of sebocyte precursors

Cellular proliferation in *Eda*^Ta^ mice was analyzed by BrdU incorporation and Ki67 immunostainings, both of which were successful as witnessed by labeling of intestinal crypts, a site of intense cell proliferation (Figure 4b). In skin, some BrdU-positive and many Ki67-positive cells were detected in the epidermis and in the pilosebaceous unit, in particular at the periphery of sebaceous glands (Figure 4c) and in hair follicles (data not shown). A single week of mAbEDAR1 treatment did not significantly change gland size, but this difference was obvious in mice treated for 3 weeks (Figure 4d). At 3 weeks, both the number and percentage of Ki67-positive cells at the periphery of sebaceous glands was higher in treated mice than in control (Figure 4d-f). Interestingly, in four weeks-old mice (at 1 week of treatment), most cells at the periphery of sebaceous glands were proliferating regardless of treatment. These data suggest that the enlargement of sebaceous glands observed after treatment is due to sustained proliferation of sebocyte precursors at the periphery of sebaceous glands.

Functional enhancement of adult sebaceous gland activity by Edar stimulation

We measured sebum output to determine whether morphologically enlarged glands can produce more sebum. Mice at weaning were treated every second week with mAbEDAR1, which we have established here to increase sebaceous gland size; mAbEDAR3, an independent Edar agonist monoclonal antibody (Kowalczyk et al., 2011); and Aprily2, a control isotype-matched monoclonal antibody (Schwaller et al., 2007) (Figure 5a). Mice treated with Edar agonists had a disheveled and greasy appearance (Figure 5b), which might be contributed to by increased sebum production from the enlarged glands. Thin layer chromatographic analyses of sebum components revealed that hair

from treated mice carried more sebum than that from control animals, this effect being particularly notable in the wax diester fraction, but also significant in the faster and slower migrating bands (Figure 5c,d). A morphological increase in sebaceous gland size is thus accompanied by increased sebum production. Effects were seen with anti-EDAR antibodies but not with a control antibody, ruling out non-specific effects of monoclonal antibody administration. At the 6 weeks time point, untreated *Eda^Ta* mice had less sebum than wild type, but this difference was no longer observed 6 weeks later (Figure 5e). Also, there was consistently more sebum in older mice (12 and 24 weeks time points) than in younger ones (6 weeks), a difference that was still visible in treated mice (Figure 5f). No significant differences were observed between sebum of males and females. Analysis by electrospray mass-spectrometry of three sebum samples per treatment condition revealed, among other lipids, the presence of lanosterol, cholesterol esters, wax monoesters (C42 to C50), a major species of wax diester (C60), mostly saturated triacylglycerols (TAGs) and O-alkyl TAGs (C50 to C62), and a prominent species of a polyunsaturated TAG (C58) (Supplementary Figures 1 and 2, and data not shown). Although treatment increased the amount of sebum, in particular wax diesters, sebum components and their fine composition were remarkably similar between wild type and *Eda^Ta* mice, before and after treatment. One exception was the polyunsaturated C58 TAG that was consistently induced by treatment in wild type but not in *Eda^Ta* mice (Supplementary Figure 1 and data not shown).

Discussion

Ectodermal dysplasias in human and in animal models are identifiable in the early postnatal period due to impairment of prenatal developmental processes. Rarely addressed is the question of whether tissue homeostasis is also controlled by EDAR pathway signaling, allowing a potential for therapeutic effects of Edar agonists administered to HED patients outside of the neonatal period. With the advent of reagents allowing time-limited modulation of the EDAR pathway (Kowalczyk-Quintas and Schneider, 2014), we can now assess adult tissue responses to modulation of this

9

pathway and their kinetics. We report that adult sebaceous glands respond to EDAR stimulation, with no apparent need for developmental stimulation to achieve this response. Indeed, in almost all cases the treated Eda^{Ta} glands attain the same size as the treated wild type glands. This homeostatic effect is slow to appear and slow to revert, and chronic treatment is required for maintenance. The half-life of mAbEDAR1 in mouse is about eleven days (Kowalczyk et al., 2011). Stability of the antibody could explain the delayed reduction in size following withdrawal, together with the cellular turnover rate of the sebaceous gland itself. Although sebaceous glands in different regions of the skin responded to Edar stimulation and withdrawal in the same manner, the magnitude of changes was different, with a relatively low limit on the amount of enlargement seen in ear skin, while dorsal and facial skin show greater increases in gland size. These regional differences in response may arise from differences in local *Eda*, *Edar* or *Edaradd* expression levels, or from the presence of different numbers of progenitor cells that can respond to the treatment in glands from different locations.

A number of Edar-responsive genes and processes acting during early hair follicle development also influence adult sebaceous gland function, potentially representing a single molecular mechanism for Edar action in both developmental and homeostatic situations. *Sonic hedgehog* expression is stimulated by Edar activity in hair follicle placodes from embryonic day 13 (Pummila et al., 2007; Schmidt-Ullrich et al., 2006), and hedgehog signaling promotes sebaceous gland development and hypertrophy (Allen et al., 2003). The function of Edar signaling to suppress BMP activity (Mou et al., 2006; Pummila et al., 2007) may also be involved as BMPs are known suppressors of sebaceous gland size (Plikus et al., 2004). As Edar agonists stimulated proliferation in sebaceous glands without increasing sebocyte size, increased proliferation of sebaceous progenitor may represent the underlying mechanism for glandular enlargement in these experiments. However, the preferential stimulation of wax diester production in sebum may additionally indicate a direct action of Edar signaling on sebocytes. Whatever the mechanism, sebaceous gland responses can serve as a sensitive biomarker for EDAR pathway stimulation in future clinical and experimental studies.

This report shows that chronic Edar stimulation in an animal model of adult XLHED has corrective effects on at least one gland type. The restoration of sebaceous function in XLHED may improve the dry and eczematous skin frequently observed in this condition (Chen, 2006; Daniel et al., 2002; Reed et al., 1970; Tyagi et al., 2011). As chronic Edar stimulation in both *Eda*-deficient and -proficient mice boosted sebum output far above levels produced by untreated wild type mice, substantial improvement of sebum production may be expected even in patients with reduced numbers of sebaceous glands. EDAR pathway stimulation may also stimulate growth and function of other glands affected by XLHED, such as salivary and/or lacrimal glands (CKQ, SS and PS, unpublished preliminary observations), to perhaps achieve functional benefit to dry eye and xerostomia. In addition, although null mutations of *EDA* lead to absence of Meibomian, eccrine and tracheal glands, some cases of XLHED likely involve hypomorphic mutations that allow rudimentary glands to form during development. For example, while *Eda*-deleted *Eda^{Ta}* mice lack sweat gland structures entirely (Gaide and Schneider, 2003; Gruneberg, 1971), a substantial fraction of people with diagnosed XLHED do have some sweat gland structures and a subset also have low level sweat production (Schneider et al., 2011). Edar agonists may stimulate these glandular rudiments to increase their function and possibly provide therapeutic benefit, as observed here for hypoplastic sebaceous glands. Indeed, a spectrum of *EDA* mutations of different severity, from null mutations causing full XLHED syndrome to mutations causing non-syndromic tooth agenesis (Mues et al., 2010; Mues et al., 2009) exist and also interact with modifier alleles in the genetic background (Cluzeau et al., 2012). Thus, stimulation of the Edar pathway in adult life may have benefit for XLHED and for other conditions affecting cutaneous glands.

Materials and Methods

Animals and antibody administration

Mice carrying the *Tabby* mutation (a deletion of the first exon and promoter region of the *Eda* gene), and their wild type controls were derived from breeder pairs of B6CBACa-*A^{w-J}*/*A-Eda^{Ta}*/O mice (000314; Jackson Laboratory) (Gaide and Schneider, 2003). *Eda^{Ta}* refers to both homozygous *Eda^{Ta/Ta}* females and hemizygous *Eda^{Ta/Y}* males. *Edar^{OVE1B/OVE1B}* mice lacking all *Edar* exons were as described (Headon and Overbeek, 1999). Anti-EDAR monoclonal antibodies mAbEDAR1 and mAbEDAR3 have been described (Kowalczyk et al., 2011). Aprily-2, a mouse IgG1 directed against human APRIL was used as a control antibody (Schwaller et al., 2007). Antibodies in sterile PBS were administered intraperitoneally at a dose of 2 mg/kg. Mice were handled according to Swiss Federal Veterinary Office guidelines, under the authorization of the Office Vétérinaire Cantonal du canton de Vaud (authorization 1370.5 to PS).

Tissue analyses and morphometrics

Hematoxylin and eosin stained 8 µm skin sections were imaged using brightfield microscopy. Features on saved microscope images were measured using ImagePro software (MediaCybernetics). The area of sebaceous glands, identified by their characteristic frothy appearance, was determined, summed for each image, and normalized to the corresponding length of skin surface to obtain the normalized aggregate gland area. At least 4 sections per animal were measured to derive a mean gland size, and each section used for measurement was at least 200 µm away from any other.

Outer and inner skins of ears were separated with forceps, washed in Hank's Balanced Salt Solution (HBSS) and incubated for 2 h in HBSS + 5 mM $CaCl_2$ + 1 mg/ml collagenase (Sigma C2674) at 37°C. Samples were then washed in PBS, fixed in 4% paraformaldehyde, rinsed in 60% isopropanol and stained in 0.5% Oil Red O in 60% isopropanol. After washing with 60% isopropanol, samples were stored in buffered formalin solution. Stereomicroscope images at the outer and inner sides of the tip of the whole mount ear samples were taken and the surface of 20 glands from each side of the ear was measured using ImagePro. A mean value for each side of the ear from each individual was calculated, and the mean of the outer and inner sides calculated to give a value for that animal. The

mean ear sebaceous gland area and standard deviation was calculated for groups of mice in each

treatment conditions.

In situ hybridization

Dorsal skin of wild type and *Edar^{OVE1B/OVE1B}* adult mice, which had been depilated 14 days earlier, was

fixed in formaldehyde, dehydrated and embedded in paraffin. Tissue sections of 5 µm were cut,

deparaffinized and processed for in situ hybridization and signal detection using the RNAScope

system according to manufacturer's instructions (Advanced Cell Diagnostics; Hayward, CA) (Wang et

al., 2012). After color detection, sections were lightly counterstained with hematoxylin and

coverslipped using an aqueous mountant.

Analysis of cell proliferation

Eda^{Ta} mice (3 mice per group) were treated intraperitoneally at weaning and 2 weeks later with

mAbEDAR1 at 2 mg/kg. Two hours prior to sacrifice (at 1 and 3 weeks after treatment initiation),

mice were given intraperitoneally BrdU at 40 mg/kg in PBS. Paraffin sections of back skin and small

intestine were immunostained with rat anti-BrdU (Gene Tex, Alton Pkwy, CA) and rabbit anti-Ki67

(Abcam, Cambridge, UK) antibodies according to standard procedures (Supplemental Material and

Methods).

Sebum analysis

Hair was shaved off the dorsum, weighed (~70 mg) and sebum extracted twice by adding 1 ml

acetone each time. The pooled acetone extracts were dried under nitrogen and sebum dissolved in 2

µl of acetone per mg of dry hair. 6 µl of each sample (equivalent to the amount of sebum in 3 mg

hair) was spotted on 20 x 5 cm HPTLC silica plates, with 3 µg of cholesterol, 1.2 µg of cholesterol

oleate, and 6 µg of rapeseed oil (triglycerides) as standards. Plates were developed twice in toluene :

n-hexane (2:1, v/v), then dried, dipped in revelation solution (10% w/v $CuSO_2.5H_2O$, 3.5% sulfuric acid, 3.1% phosphoric acid in methanol) and heated at 140°C until appearance of bands. The plate was then photographed. Intensities of rapidly (including cholesterol esters and wax monoesters), intermediate (wax diesters) and slowly (including cholesterol and triglycerides) migrating bands were measured using the ImageJ software. Animals used for sebum determination were a separate set from those used for measurement of sebaceous gland size.

Statistics

Statistical analysis was performed with the Prism software using unpaired t test for the comparison of two samples and ANOVA with Newman-Keuls multiple comparison test for multiple samples.

Conflict of interest

PS is shareholder of Edimer Pharmaceuticals. NK is shareholder, director and employee of Edimer Pharmaceuticals. DJH has acted as a consultant for Edimer Pharmaceuticals. KH is an employee of Edimer Pharmaceuticals.

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Figure legends

Figure 1. Edar stimulation in adult mice increases facial sebaceous gland size. (**a**) Schematic of experimental schedule using mAbEDAR1. Animal age is indicated above. Gray color indicates no Edar stimulation. (**b**) H&E stained sections of facial skin of untreated wild type and *Eda^Ta* mice, and of *Eda^Ta* mice continually treated for 5 or 6 months, with analysis at 6 months. Right panels show details of sebaceous glands (red dashed lines). Glands are smaller in *Eda^Ta* than wild type. Both treatments rescue gland size. Scale bars indicate 100 μm. (**c**) Quantification of sebaceous gland size on sectioned facial tissues. (**d**) Quantification of sebocyte size as defined by number of nuclei per unit sebaceous gland area. This is unchanged by condition. Error bars indicate standard deviation.

Figure 2. Chronic Edar stimulation rescues *Eda^Ta* ear sebaceous glands. (**a**) Schematic of experimental schedule. Weeks since beginning of treatment are indicated above. (**b**) Oil-Red-O staining of sebaceous glands in ear skin. Conditions as indicated. Scale bar: 250 μm. Enlarged insets have edge lengths of 250 μm. (**c**) Sebaceous gland size in untreated *Eda^Ta* and wild type. (**d**) Effects of mAbEDAR1 administration on sebaceous gland size of *Eda^Ta* mice. Untreated gland sizes as in panel c. By 6 weeks post-treatment gland size is increased. Sustained treatment is required to achieve full rescue. (**e**) Effects of mAbEDAR1 treatment on wild type sebaceous glands. Untreated gland sizes are as in panel c. Adjacent data bars with no p-value indicate p>0.05. Error bars indicate standard deviation.

Figure 3. Chronic stimulation of Edar signaling greatly stimulates sebaceous gland enlargement in *Eda^Ta* and wild type dorsal skin. (**a**) Hematoxilin and eosin-stained tissue sections of dorsal skin of *Eda^Ta* and wild type mice either untreated, treated once only at weaning or treated every 14 days from weaning for 12 weeks. Insets show examples of sebaceous glands at higher magnification. Scale bars indicate 100 μm. (**b**) Quantification of aggregate sebaceous gland size per unit length of dorsal

skin. (**c**) Same as panel b, but for ear skin at 12 weeks processed in the same way. Error bars indicate standard deviation.

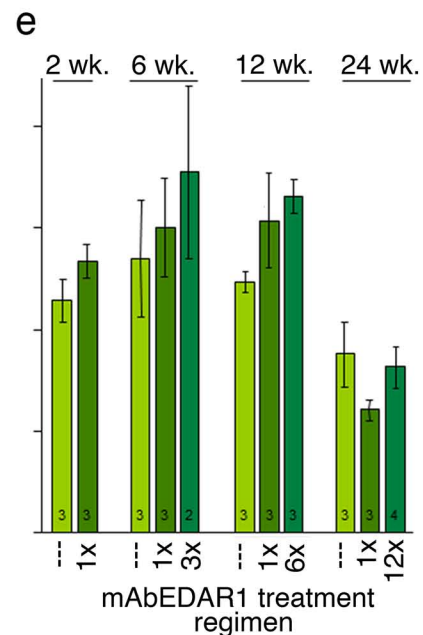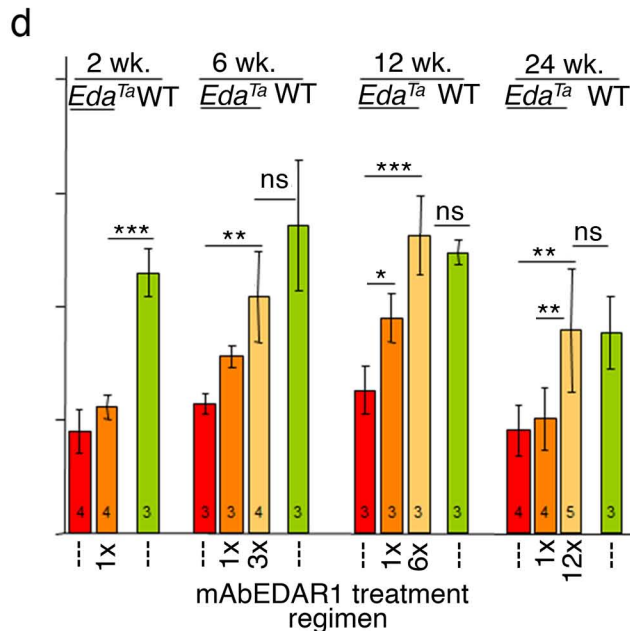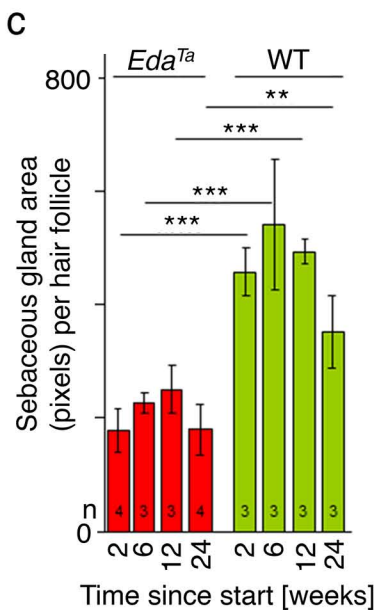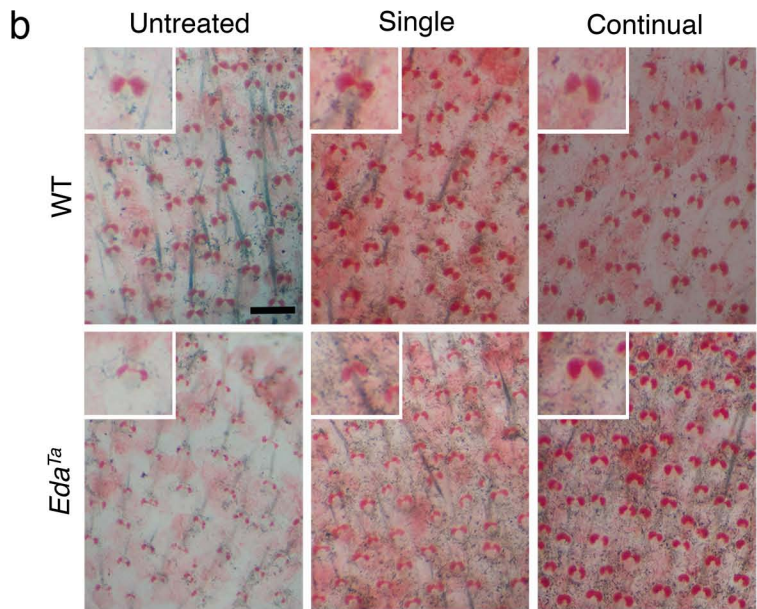**Figure 4. *Edar* expression and cell proliferation in adult sebaceous glands.** (**a**) Skin sections of the indicated mice with hair follicles in anagen phase were hybridized with the indicated probes. *Edar* transcripts in sebaceous glands are detected as red dots (black arrows), but none is detected in *Edar*$^{OVE1B/OVE1B}$ glands. (**b-c**) Ki67 and BrdU immunostainings of small intestine villi (b) and representative sebaceous glands (highlighted by dotted red lines) in adjacent sections of skin of mice treated for 3 weeks ± anti-EDAR mAb (c). (**d-f**) Quantification of gland area (d), number of Ki67-positive cells at the periphery of sebaceous glands (e), and percentage of cells that are Ki67-positive at the periphery of sebaceous glands (f) in *Eda*^{Ta} mice treated as indicated. Bars indicate mean ± SD.

Figure 5. Edar activation stimulates sebum production. (**a**) Schematic of experimental schedule. (**b**) Appearance of 9 weeks-old *Eda*^{Ta} mice treated with EDAR agonist antibodies, PBS or the unrelated antibody Aprily2. (**c**) Thin layer chromatography analysis of sebum extracted from dorsal hair. CE, cholesterol ester; WDE, wax diester; TG, triglyceride; C, cholesterol. (**d**) Quantification of wax diester band intensity. Heavy bands are underestimated (non-linear response). n: animals per condition. (**e**) Total sebum quantification of unstimulated WT and *Eda*^{Ta} mice. (**f**) Quantification of total sebum at 6 weeks or 12 to 24 weeks (> 6 w) treatment in unstimulated (Ctrls) or stimulated (mAbs) mice. WT and *Eda*^{Ta} (Tb) values are pooled. *, $p < 0.05$. ns, not significant. ***, $p < 0.001$. Error bars indicate SEM.

a
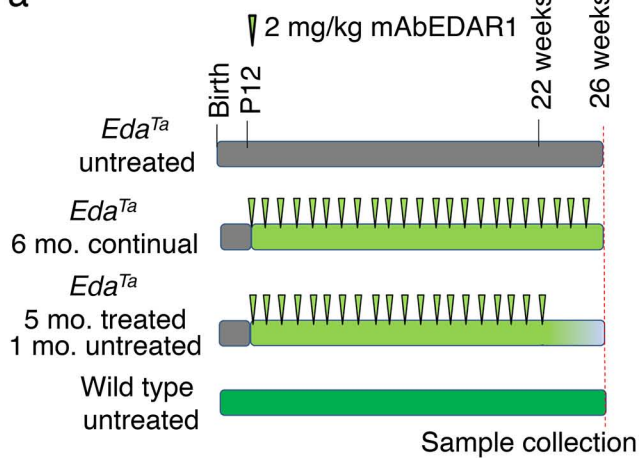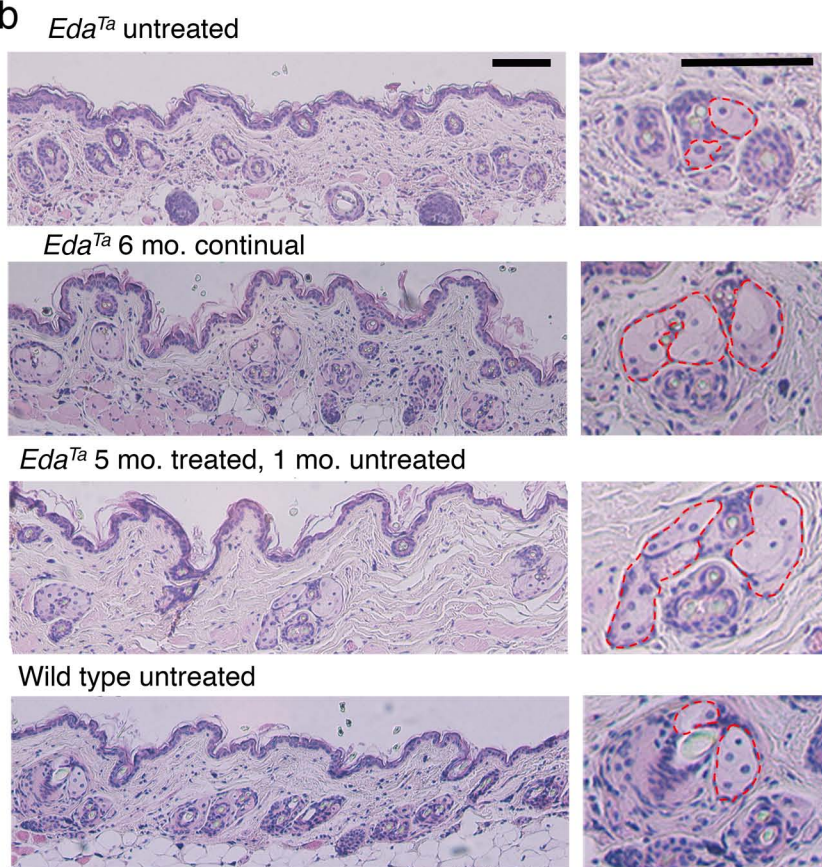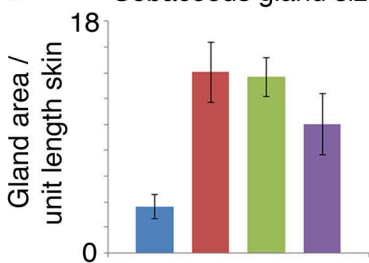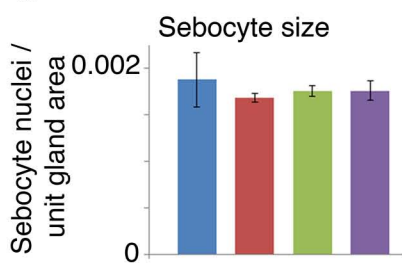
2 mg/kg mAbEDAR1

Birth
P12
22 weeks
26 weeks

Eda^Ta
untreated

Eda^Ta
6 mo. continual

Eda^Ta
5 mo. treated
1 mo. untreated

Wild type
untreated

Sample collection

b

Eda^Ta untreated

Eda^Ta 6 mo. continual

Eda^Ta 5 mo. treated, 1 mo. untreated

Wild type untreated

c

Sebaceous gland size

Gland area / unit length skin

18

0

d

Sebocyte size

Sebocyte nuclei / unit gland area

0.002

0

Eda^Ta
untreated

Eda^Ta
6 mo. continual

Eda^Ta
5 mo. treated
1 mo. untreated

Wild type
untreated

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a *Eda^{Ta}* back skin — Wild type back skin

Untreated

Single

Continual

b Back skin sebaceous gland size

12 weeks — 24 weeks

Gland area/ unit skin length

** **

untreated single continual | untreated single continual

Eda^{Ta} | Wild type

*** ***

untreated single continual | untreated single continual

Eda^{Ta} | Wild type

c Ear skin sebaceous gland size, 12 weeks

*

untreated continual | untreated continual

Eda^{Ta} | Wild type

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a ISH adult sebaceous glands

Mice	Probe	
Wild type	*Edar*	
Edar^OVE1B	*Edar*	
Wild type	*dapB* (neg. control)	

50 μm

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b Intestine

Ki67

BrdU

Ki67

BrdU

50 μm

c Back skin

Eda-deficient, PBS, 3 weeks

Eda-deficient, mAbEDAR1, 3 weeks

50 μm

d

Gland size [pixels]

200 k

ns

***

0

e

# of Ki67+ cells at gland periphery

12

ns

***

0

f

% of Ki67+ cells at gland periphery

100

ns

***

0

PBS 1 W mAb1 1 W PBS 3 W mAb1 3 W

a

Birth | P21-P26 | 6 weeks | 12 weeks | 24 weeks

Hair collection for sebum extraction

❚ : 2 mg/kg antibody administration
Same mice used for 6 and 24 weeks time points

b

Eda^{Ta} (*Eda*-deficient; Tabby; Tb)   6 weeks

mAbEDAR1 [mAb1]   mAbEDAR3 [mAb3]   Aprily2 [Ctrl]   PBS [---]

c

Eda^{Ta} (*Eda*-deficient; Tabby; Tb)   WT

Females   Males   Females   Males

--- mAb1 mAb3 Ctrl --- mAb1 mAb3 Ctrl mAb1 mAb3 Ctrl --- mAb1 mAb3 Ctrl

C+CE TAG

CE
WDE
TAG
C

6 weeks

12 weeks

not available

24 weeks

d

All controls vs all treated. p < 10e-19

WDE band intensity [arbitrary units; AU]

*** *** *** *** ***

4 4 4 4 | 4 4 4 4 | 4 4 4 4 | 3 4 4 4 | 4 4 4 4   n

--- Ctrl mAb1 mAb3

Tb 6w | Tb 12w | Tb 24w | WT 6w | WT 12w

e

All bands intensity [AU]

* ns

8 8 | 8 8   n

Tb WT | Tb WT
6 w | 12 w

f

All bands intensity [AU]

*** *** ***

16 23 | 16 24   n

6 w > 6 w | 6 w > 9 w
Ctrls | mAbs

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