Pharmacological Stimulation of Edar Signaling in the Adult Enhances Sebaceous Gland Size and Function

Christine Kowalczyk-Quintas[1], Sonia Schuepbach[1], Laure Willen[1], Terry K. Smith[2], Kenneth Huttner[3], Neil Kirby[3], Denis J. Headon[4,#] and Pascal Schneider[1,#]

[1] Department of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland. [2] Biomedical Sciences Research Complex, University of St Andrews, North Haugh, St Andrews, Fife, KY16 9ST, UK. [3] Edimer Pharmaceuticals, Cambridge, MA. [4] Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin EH25 9RG, UK. # Shared senior authorship.

Running title: EDAR in adult sebaceous glands.

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

Authors for correspondence: pascal.schneider@unil.ch; denis.headon@roslin.ed.ac.uk

Pascal Schneider, Department of Biochemistry, University of Lausanne, Boveresses 155, CH-1066 Epalinges, Switzerland; Phone: +41 21 692 5709; Fax: +41 21 692 5705

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, and it is unknown whether EDAR stimulation in adults with XLHED might have beneficial effects. We find that sebaceous glands of adult *Eda* mutant mice enlarge slowly when administered an agonist anti-EDAR antibody, are maintained in an enlarged state upon chronic treatment, but regress slowly over a period of weeks 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, Phillips et al. 1987, Wright, Grange 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, Srivastava et al. 1996), or caused by mutation of the *EDAR* gene itself (Monreal, Ferguson et al. 1999) or affecting its intracellular adapter protein EDARADD (Headon, Emmal 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, Smahi et al. 2001, Schmidt-Ullrich, Aebischer 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, Skovbo 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 (Reed, Lopez et al. 1970, Tyagi, Tyagi et al. 2011, Dietz, Kaercher et al. 2013), administering artificial saliva due to xerostomia arising from salivary gland reduction (Daniel, McCurdy et al. 2002), removal of nasal and otic crusting (Callea, Teggi et al. 2013) and application of emollient to improve dry skin and eczema symptoms (Chen 2006, Tyagi, Tyagi et al. 2011). External cooling during physical exertion or in hot weather may also be needed due to reduced or absent sweating (Hammersen, Neukam et al. 2011, Schneider, Hammersen et al. 2011).

Since the identification of the EDAR pathway, a number of reagents to modulate its signaling have been developed. Pathway stimulation can be achieved using a modified form of recombinant EDA consisting of its C-terminal TNF domain fused to an immunoglobulin Fc domain (Gaide and Schneider 2003). Acting in a manner analogous to that of the endogenous ligand, monoclonal antibodies that bind the EDAR extracellular domain also stimulate this pathway (Kowalczyk, Dunkel et al. 2011), presumably by causing clustering and activation of the receptor, while suppression of the EDA signal may be achieved by blocking monoclonal antibodies directed against the ligand's receptor binding domain (Kowalczyk-Quintas, Willen 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 (Srivastava, Pispa et al. 1997, Headon and Overbeek 1999, Headon, Emmal et al. 2001). The morphological defects caused by *Eda* mutation can be rescued by prenatal or perinatal administration of recombinant Fc-EDA protein (Gaide and Schneider 2003, Casal, Lewis et al. 2007), or ligand replacement using an EDAR agonist antibody (Kowalczyk, Dunkel 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, Mao-Qiang et al. 2003, Fluhr, Darlenski et al. 2008), modulate the skin microflora (Fischer, Blanchette et al. 2013), deliver antioxidants to the skin surface (Thiele, Weber et al. 1999) and maintain the hair follicles (Zheng, Eilertsen et al. 1999, Sundberg, Boggess et al. 2000, Stenn 2001). Altered sebaceous gland activity is implicated in the pathogenesis of a number of dermatological conditions, including acne vulgaris (Zouboulis, Jourdan et al. 2013), some forms of dermatitis (Gary 2013) and rosacea (Raghallaigh, Bender et al. 2012),

while reduced sebaceous function may be causative in hidradenitis suppurativa (Kamp, Fiehn et al. 2011) and scarring alopecia (Al-Zaid, Vanderweil 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 (Reed, Lopez et al. 1970, Daniel, McCurdy et al. 2002). 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, Durmowicz et al. 2003, Mustonen, Pispa et al. 2003). Cui et al. (Cui, Durmowicz 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, Dunkel et al. 2011, Kowalczyk-Quintas, Willen 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 there is a detectable morphological response of sebaceous glands to administration of the EDAR agonist monoclonal antibody mAbEDAR1 (Kowalczyk, Dunkel 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). 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, with an aggregate sebaceous gland size less than half that 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. In order to determine the basis of increased sebaceous gland size in the treated animals, we measured sebocyte size in all conditions (Figure 1d). This parameter was unchanged across treatments and genotypes, 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 measure sebaceous gland size. This involved visualization of sebaceous glands in ear skin using collagenase 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).

Measurement of average sebaceous gland size per hair follicle in the ear revealed that gland size is smaller in Eda^{Ta} than wild type animals across the experimental time course from 2 to 24 weeks post-weaning (Figure 2c), as expected. Gland sizes are relatively stable at different ages in adult skin, though with a trend to reduced size in older individuals. Treatment with mAbEDAR1 did not have a 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 only displayed 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 not anymore at 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

To determine whether sebaceous glands in the more intensively studied dorsal skin respond in the same manner as in ear skin to mAbEDAR1 administration, we analyzed gland size in skin sections at the 12 weeks time point. Clear differences between continually treated and single and untreated animals in the size of the sebaceous glands were apparent in the sections (Figure 3a). We determined aggregate sebaceous gland size in Eda^{Ta} skin to be about half that of wild type, but chronic treatment with mAbEDAR1 stimulates a large increase in the size of both mutant and wild

type glands. Similar to what was observed in the ear, a single administration of mAbEDAR1 was less efficient than chronic treatment to increase sebaceous gland size 12 or 24 weeks later (Figure 3b). The response in dorsal skin appears less limited than in the ear skin as we measured at 12 weeks 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 the mAbEDAR1 monoclonal antibody, 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 type animals but not in $Edar^{OVE1B/OVE1B}$ mice lacking all *Edar* exons (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

Eda-deficient mice that were treated for one or three weeks with buffer only or mAbEDAR1 were given BrdU two hours before sacrifice. Proliferation-specific Ki67 and BrdU stainings were successful as witnessed by labeling of intestinal crypts, a site of intense cell proliferation (Figure 4b). In skin, some BrdU-positive and a greater number of Ki67-positive cells were detected in the epidermis, in hair follicles (data not shown) and in the pilosebaceous unit, in particular at the periphery of sebaceous glands (Figure 4c). In line with our previous results, after one week of treatment, there was no significant change in 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,e). 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 may be due to sustained proliferation of sebocyte precursors at the periphery of sebaceous glands.

Functional enhancement of adult sebaceous gland activity by Edar stimulation

In order to determine whether the morphological increase in sebaceous glands is accompanied by increased function, we determined sebum output in treated and untreated Eda^{Ta} and wild type mice. Mice at weaning were treated every second week for 6, 12 or 24 weeks with mAbEDAR1, which we have established here to increase sebaceous gland size; mAbEDAR3, an independent Edar agonist monoclonal antibody (Kowalczyk, Dunkel et al. 2011); and Aprily2, an unrelated isotype-matched monoclonal antibody serving as a negative control (Schwaller, Schneider et al. 2007) (Figure 5a). First, we noted that mice treated with Edar agonists have a disheveled and greasy appearance (Figure 5b), which might be contributed to by increased sebum production from the enlarged glands. We then assayed sebum deposition onto the dorsal hairs in treated and untreated animals using thin layer chromatography to separate sebum components. Chromatography revealed that hair from mAbEDAR1- and mAbEDAR3-treated animals carries more sebum than that from untreated or Aprily2-treated animals, this effect being particularly notable in the wax diester fraction, but also significant in the faster and slower migrating bands (Figure 5c,d). This confirms that the morphological increase in sebaceous gland size is accompanied by increased sebum production, and this results from Edar pathway stimulation rather than a non-specific effect of monoclonal antibody administration. At the 6 weeks time point, untreated *Eda*-deficient 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 per 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 polyunsaturated TAG (C58) (Supplementary Figures 1 and 2). Although treatment clearly increased the amount of sebum, sebum components and their fine composition were remarkably similar between wild type and *Eda*-deficient mice, before and after treatment, suggesting that EDAR stimulation increases the number of sebocytes more than their function. One exception was the polyunsaturated C58 TAG that, although present in untreated *Eda*-deficient mice, was not induced by treatment in the three independent samples analyzed (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 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. A developmental effect that might reduce sebaceous gland size in XLHED, such as laying out of a smaller number of responding progenitor cells in prenatal skin, does not appear to occur and in almost all cases the treated Eda^{Ta} glands attain the same size as the treated wild type glands. We went on to define the kinetics of this morphological response, finding that this homeostatic effect is slow to appear and slow to revert, but that chronic treatment is required for maintenance. The half-life of mAbEDAR1 in mouse, as measured by its ability to bind EDAR, is estimated to be eleven days (Kowalczyk, Dunkel et al. 2011), this protein stability presumably having some impact on 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* or *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 genes and processes known to be targeted by Edar signaling during early hair follicle development are also known to 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 (Schmidt-Ullrich, Tobin et al. 2006, Pummila, Fliniaux et al. 2007), and hedgehog signaling is known to promote sebaceous gland development and hypertrophy (Allen, Grachtchouk et al. 2003). The function of Edar signaling to suppress BMP activity (Mou, Jackson et al. 2006, Pummila, Fliniaux et al. 2007) may also be involved as BMPs are known suppressors of sebaceous gland size (Plikus, Wang et al. 2004). As sebocyte size is not affected by Edar stimulation, the change in balance of these, and perhaps other signals, is likely to promote sebaceous progenitor proliferation as an underlying mechanism for glandular enlargement in these experiments, in line with the observed increased percentage of Ki67-positive cells in stimulated glands. 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 (Reed, Lopez et al. 1970, Daniel, McCurdy et al. 2002, Chen 2006, Tyagi, 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 due to reduced hair follicle density. It is also possible that EDAR pathway stimulation is capable of stimulating growth and function of other glands affected by XLHED for therapeutic effect. Thus glands present but reduced in function in XLHED, such as salivary and lacrimal glands, may respond in a similar manner to EDAR stimulation in the adult period to achieve functional benefit to dry eye and xerostomia (CKQ, SS and PS, unpublished preliminary observations). In addition, although null mutations of *EDA* lead to absence of Meibomian, eccrine and tracheal glands, some cases of XLHED likely involve strongly hypomorphic mutations that allow a rudimentary gland to form during development. For example, while the Eda^{Ta} mouse carrying a deletion in *Eda* has no sweat gland structures at all (Gruneberg 1971, Gaide and Schneider 2003), a substantial fraction of people with diagnosed XLHED do have some sweat gland structures and a subset also have low level sweat production (Schneider, Hammersen et al. 2011). Such glandular rudiments may not be sufficient to allow full physiological activity, but may be brought to increased function and therapeutic benefit by chronic pharmacological EDA pathway activation, analogous to the responses of the hypoplastic sebaceous glands reported here. Indeed, it is emerging that a spectrum of *EDA* mutations of different severity, from null mutations causing full XLHED syndrome to mutations causing non-syndromic tooth agenesis (Mues, Griggs et al. 2009, Mues, Tardivel et al. 2010) exist and also interact with modifier alleles in the genetic background (Cluzeau, Hadj-Rabia 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, which is 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-$A^{w\text{-}J}$/*A*-Eda^{Ta}/O mice (000314; Jackson Laboratory) (Gaide and Schneider 2003). Eda^{Ta} is used here to refer 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). Generation and characterization of anti-EDAR monoclonal antibodies has been described (Kowalczyk, Dunkel et al. 2011). Aprily-2, a mouse IgG1 directed against human APRIL, with no cross-reaction to mouse APRIL, was used as a control antibody (Schwaller, Schneider 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

Formalin-fixed skin tissues were dehydrated, processed and embedded in paraffin and sectioned at 8 μm thickness. After drying, slides were stained with hematoxylin and eosin, coverslipped and imaged using brightfield microscopy. Features on saved microscope images were measured using ImagePro software (MediaCybernetics). Sebaceous glands were identified by their characteristic frothy appearance and were circumscribed to determine their area in pixels, which was summed for each image. The corresponding length of skin surface for that image was measured in pixels and used to normalize aggregate gland area. At least 4 sections were measured to derive a mean gland size for that individual animal and each section used for measurement was at least 200 μm away from any other.

Ear skin was processed for whole mount visualization of sebaceous glands by collection of ears followed by separation of the outer and inner skins using forceps. Skin samples were 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 then stained in 0.5% Oil Red O in 60% isopropanol. After washing with 60% isopropanol, samples were stored in buffered formalin solution. Stereomicroscope images of the outer and inner sides of the tip of the ear samples were taken and 20 glands from each side of the ear were measured in pixels by circumscribing the glands on the images 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, Flanagan 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 *i.p.* 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 *i.p.* BrdU at (Sonia, Christine: amount, conc, volume, vehicle??). Back skin and small intestine were fixed, embedded in paraffin and immunostained with anti-BrdU (which?) or anti-Ki67 (which?) antibodies ((some details??)).

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.

Electrospray-mass spectrometry analysis

Lipid extracts were dissolved in 15 µl of chloroform : methanol (1:2) and 15 µl of acetonitrile : iso-propanol : water (6:7:2) and analyzed with a Absceix 4000 QTrap, a triple quadrupole mass spectrometer equipped with a nanoelectrospray source. Samples were delivered by direct infusion mode (~125 nl/min) and analyzed in both positive and negative ion modes using a capillary voltage of 1.25 kV. MS/MS scanning (daughter, precursor and neutral loss scans) were performed using nitrogen as the collision gas with collision energies between 35-90 V. Each spectrum encompasses at least 50 repetitive scans. Tandem mass spectra (MS/MS) *i.e.* daughter ion scanning was performed with collision energies between 35-90 V. Assignment of species is based upon a combination of survey, daughter, precursor and neutral loss scans. The identity of cholesterol esters and triacylglycerol species were verified using the LIPID MAPS: Nature Lipidomics Gateway (www.lipidmaps.org).

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 absence of 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. Details of sebaceous glands (red dashed lines) are shown on the right. Glands are smaller in *Eda*^{Ta} than wild type. Both continual and 1-month ceased treatment fully rescues 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.

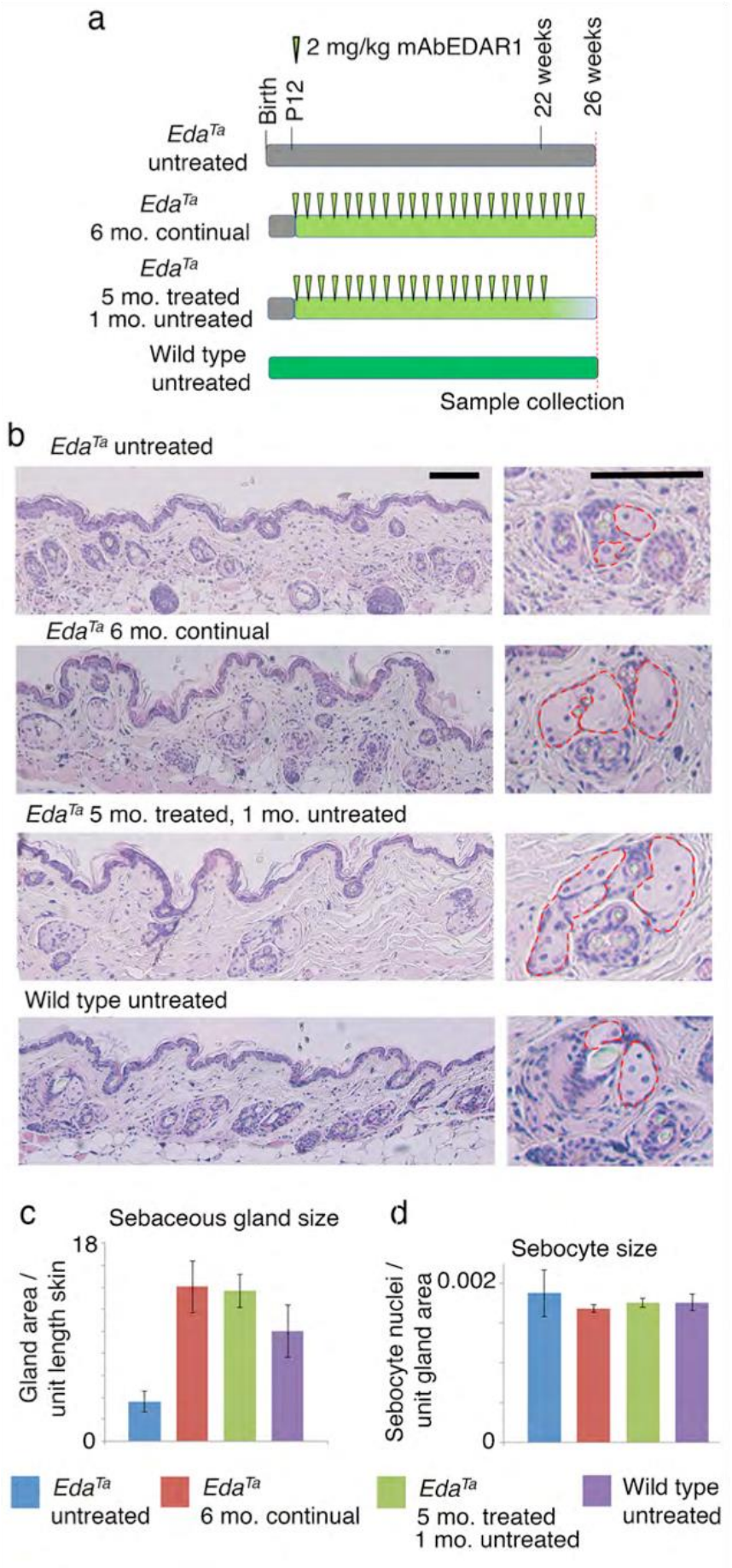
Figure 2. Chronic Edar stimulation rescues *Eda*^{Ta} ear sebaceous glands. (**a**) Schematic of experimental schedule. Weeks since beginning of treatment are indicated above. (**b**) Oil-Red-O staining of sebaceous glands in ear skin. Conditions as indicated. Scale bar: 250 μm. Enlarged insets have 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**) H&E 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

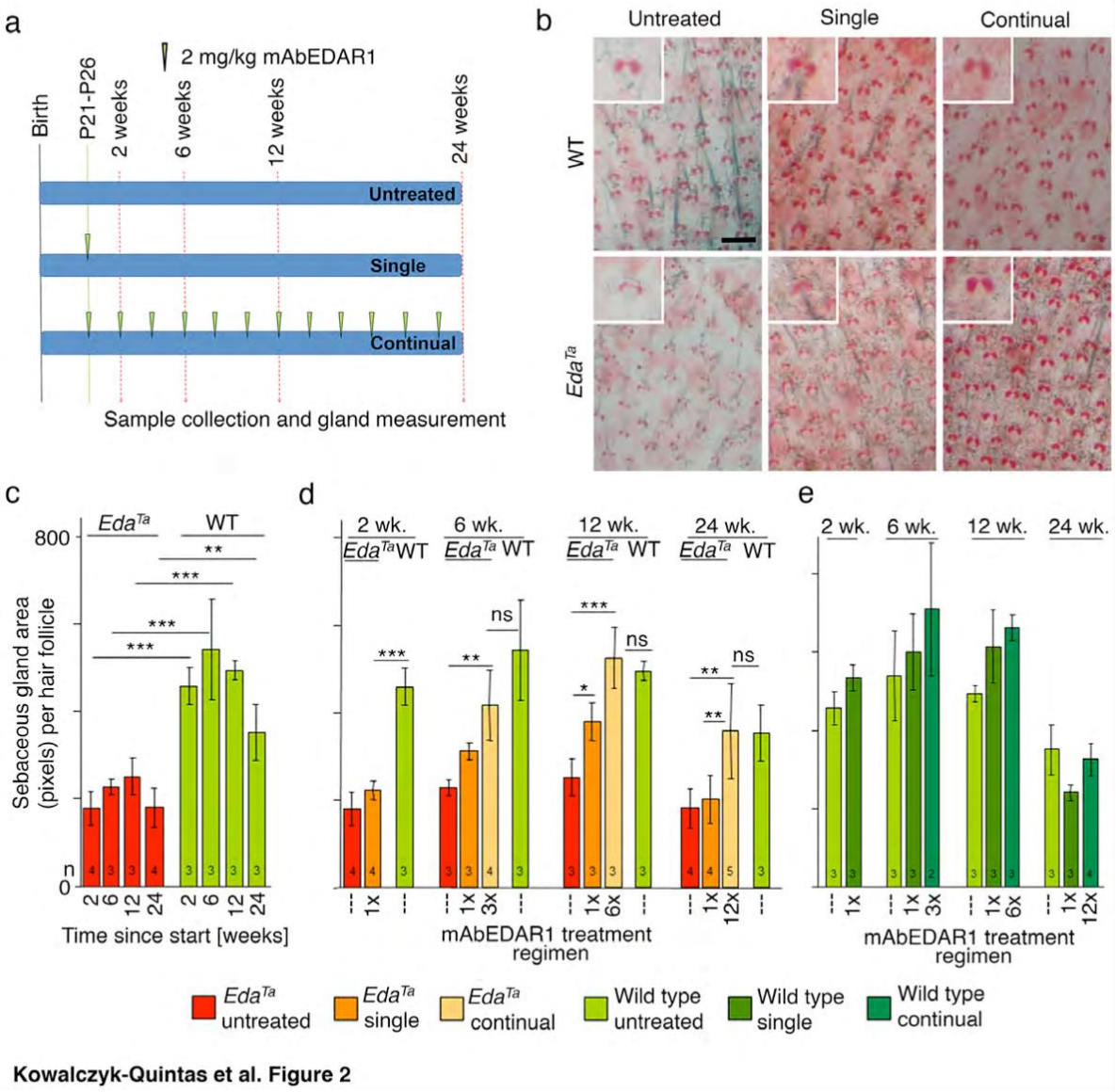
μm. (**b**) Quantification of aggregate sebaceous gland size per unit length of dorsal skin. (**c**) Same as panel b, but for ear skin at 12weeks processed in the same way. Error bars indicate standard deviation.

Figure 4. *Edar* expression in adult skin. (a) Skin sections of the indicated mice with hair follicles in anagen phase were hybridized with *Edar* probe or with the negative control probe set (targeting the *dapB* gene from *B. subtilis*). *In situ* hybridization detects *Edar* transcripts as red dots at the periphery of wild type, but not *Edar*-deficient ($Edar^{OVE1B/OVE1B}$) sebaceous glands (black arrows). A negative control probe set (targeting the *dapB* gene from *B. subtilis*) does not yield signal around the sebaceous glands in wild type skin. (b) Ki67 and BrdU immunostainings of small intestine villi. (c) Ki67 and BrdU immunostainings of representative sebaceous glands (highlighted by the dotted red line) in adjacent sections of skin of mice treated or not for 3 weeks with anti-EDAR mAb. (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*-deficient mice treated for 1 or 3 weeks with anti-EDAR antibody mAbEDAR1 or with PBS.

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.



Kowalczyk-Quintas et al. Figure 1



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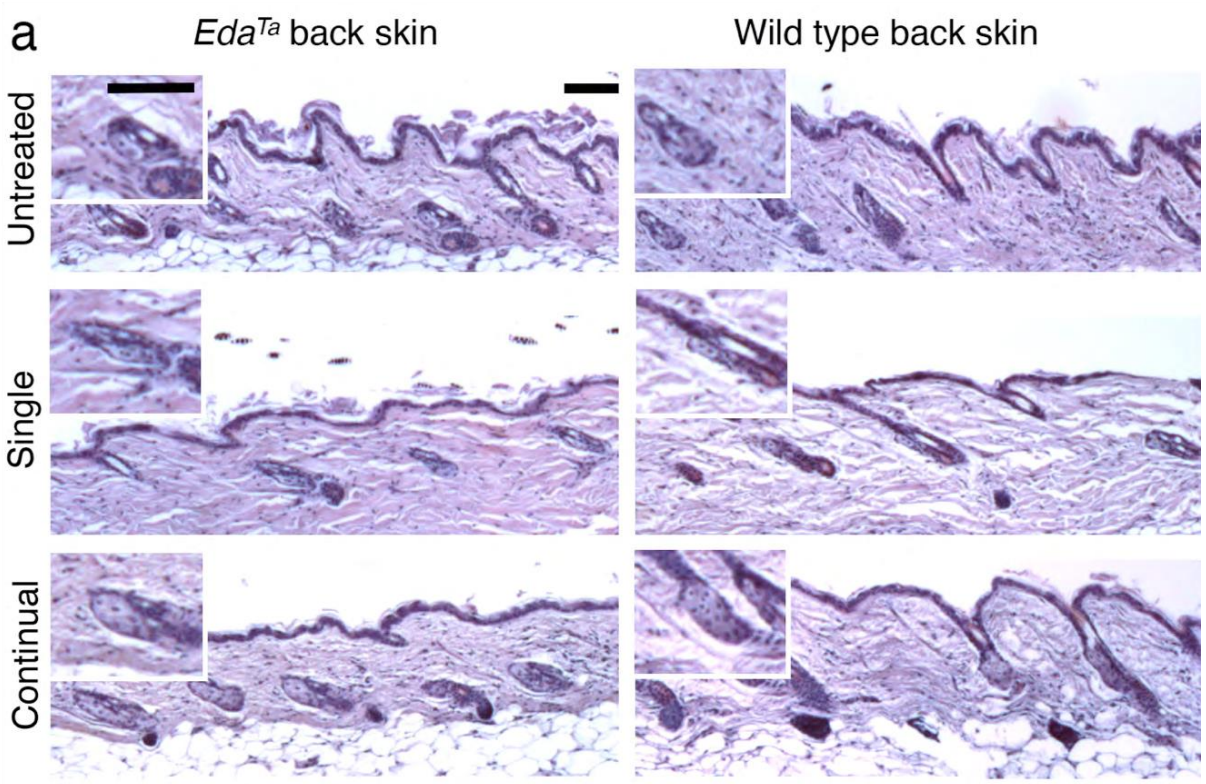

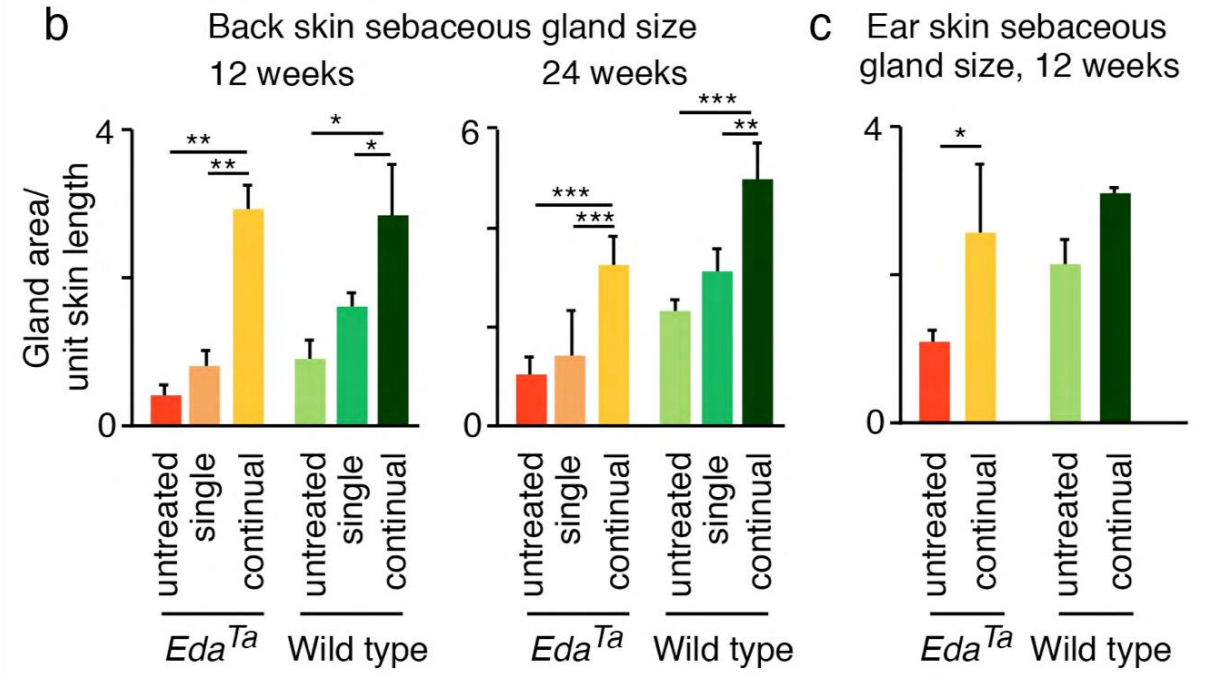

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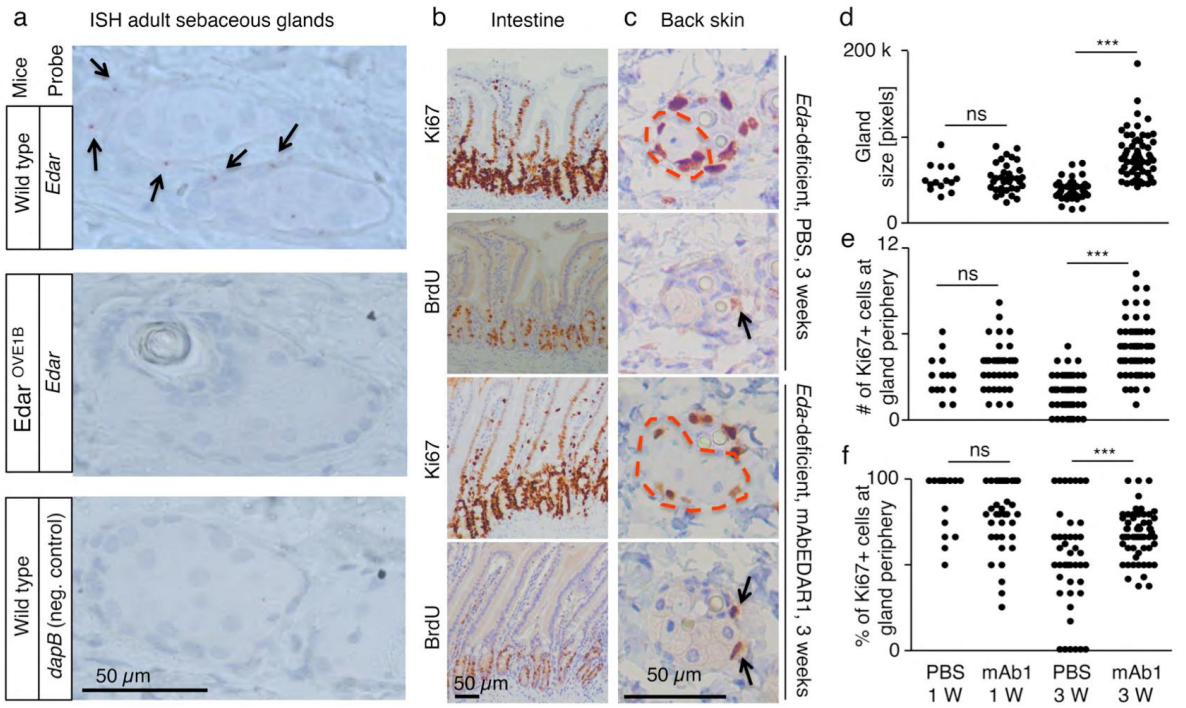

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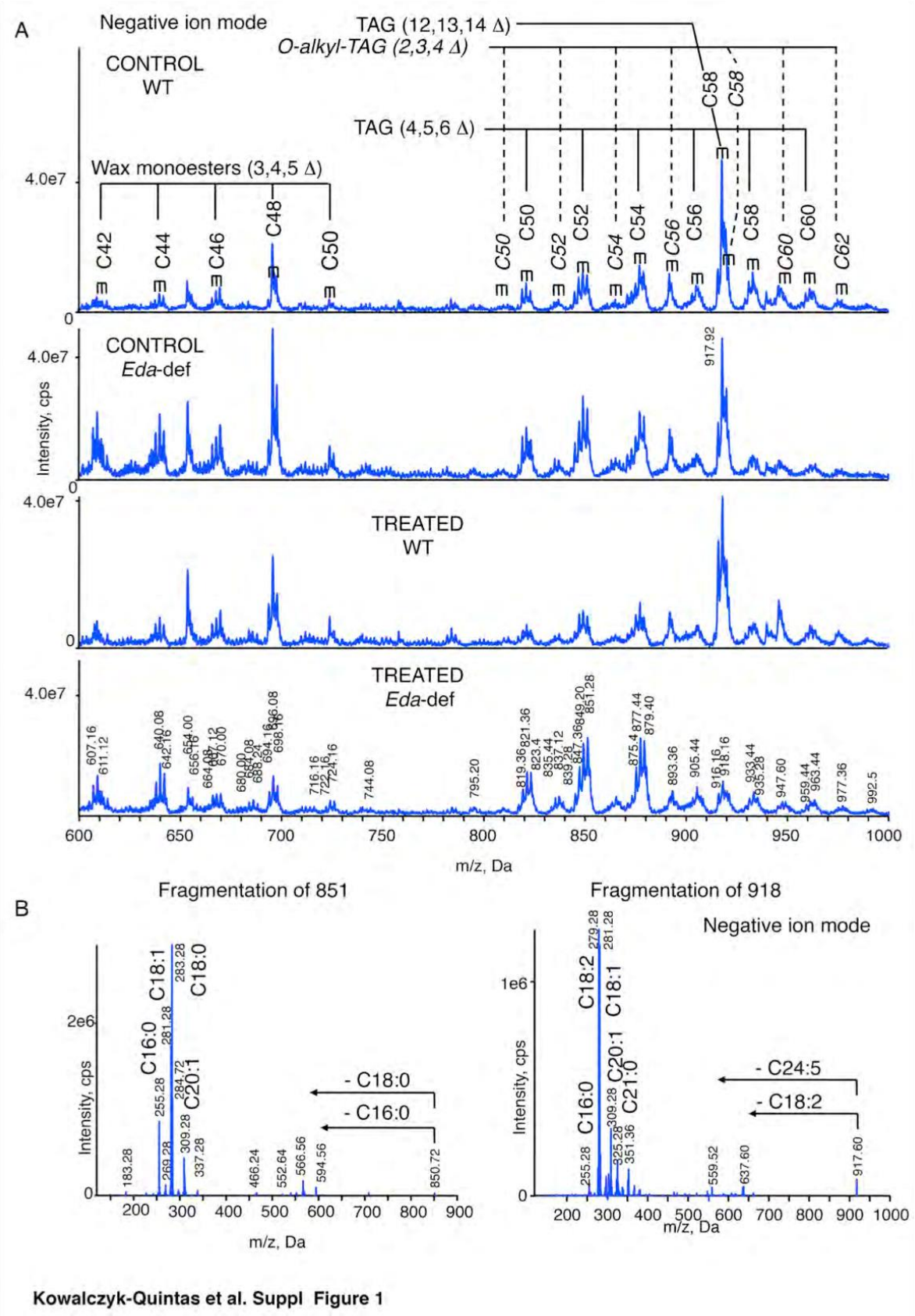

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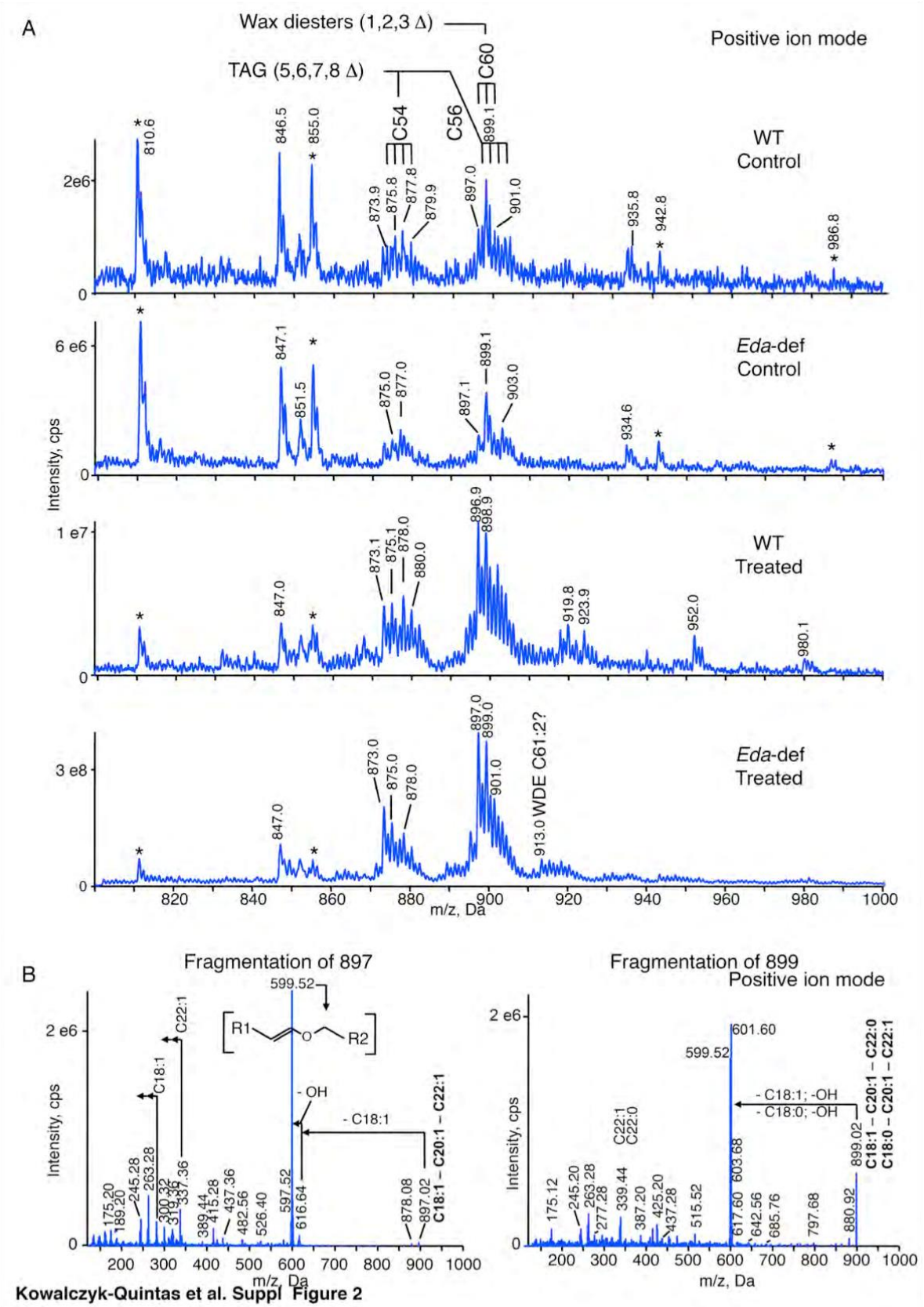

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