Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development

Johanna Pispa1,†, Marja Pummila1,†, Philip A. Barker2, Irma Thesleff1 and Marja L. Mikkola1,*

1Developmental Biology Program, Institute of Biotechnology, 00014 University of Helsinki, PO Box 56, Finland and
2Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

Received June 19, 2008; Revised and Accepted August 5, 2008

The development of ectodermal organs requires signalling by ectodysplasin (Eda), a tumor necrosis factor (TNF) family member, its receptor Edar and downstream activation of the nuclear factor kappaB (NF-κB) transcription factor. In humans, mutations in the Eda pathway components cause hypohidrotic ectodermal dysplasia, a syndrome characterized by missing teeth, sparse hair and defects in sweat glands. It has been postulated that Eda acts redundantly with another TNF pathway to regulate ectodermal organogenesis. A potential candidate is Troy (or TNFRSF19 or Taj), a TNF receptor which is homologous with Edar in its ligand-binding domain, and is expressed in an overlapping pattern. We have characterized Troy null mice and crossed them with Eda-deficient mice. Single Troy mutants had no defects in ectodermal organs. Analysis of the double mutants revealed an essential role for Troy in hair follicle development. In mice, hair follicles develop in three different waves. Only primary hair follicles are missing in Eda single mutants, whereas the compound mutants lacked also the follicles of the second wave, as well as all hair follicles in the middle of crown leading to focal alopecia. Assessment of NF-κB activity with a transgenic reporter construct indicated that Eda is the main activator of NF-κB signalling in developing skin appendages and surprisingly that the functional overlap of Troy and Eda signalling pathways is mediated by NF-κB independent pathways.

INTRODUCTION

Skin appendages, such as hair, teeth and several exocrine glands originate from the ectoderm. Their development is initiated during embryogenesis by the formation of an epithelial thickening, a placode. Subsequently, mesenchymal cells aggregate beneath the placode, which will then proliferate to form a bud-like structure. Further development includes organ-specific growth and differentiation of both epithelial and mesenchymal cells (1–3).

The inductive interactions driving ectodermal organogenesis are mediated by members of the transforming growth factor β, fibroblast growth factor, Wnt, hedgehog (Hh) and Notch ligand signalling factor families (2,4). Development of skin appendages requires also ectodysplasin (Eda), a tumor necrosis factor (TNF) family ligand and Edar, the receptor of the Eda-A1 isoform of ectodysplasin. Mutations in genes encoding Eda, Edar or the cytosolic Edar-specific signal mediator Edaradd result in hypohidrotic ectodermal dysplasia (HED) (MIM 305100, 224900 and 129490) (5,6). HED is characterized by missing and malformed teeth, sparse hair and defects in a number of glands including lacrimal, mammary and sweat glands. In mice, corresponding mutations result in highly similar ectodermal defects (7,8). Signalling by the Eda-A2 isoform of ectodysplasin is thought to be dispensable for development as mice deficient for its receptor Xedar have no apparent abnormalities (9).

The downstream signalling pathways of Eda-A1/Edar have been studied in some detail. So far, available evidence suggests that Edar, like many other TNFRs (10), signals via the transcription factor complex nuclear factor kappaB (NF-κB) and that NF-κB activity is required for ectodermal organogenesis. Canonical NF-κB activation involves phosphorylation of IκB, an inhibitor of NF-κB, by the IκB kinase (IKK) complex. This leads to degradation of IκB allowing the translocation of NF-κB from the cytosol to the nucleus and transcription of target genes (11). Edar has been shown to activate NF-κB in vitro (12–14), and suppression of NF-κB signalling by overexpressing a degradation resistant IκBα in transgenic mice (cIκBα mice) results in...
an ectodermal phenotype very similar to that observed in *Eda* (Tabby), *Edar* (downless) and *Edaradd* (crinkled) mutant mice (15). Hypermorphic mutations in IkBα cause ectodermal dysplasia in humans (16,17). Furthermore, IKKγ (or NEMO), an obligate component of the IKK complex, is essential for Edar signalling (18) and hypomorphic mutations in it lead to HED-like ectodermal defects in humans (18,19). Thus, it is generally thought that the *in vivo* functions of Eda-A1/Edar pathway are mediated largely by NF-κB activation.

The hair and tooth phenotypes of *Eda* (lacking both isoforms) and *Edar* mutant mice have been described in detail and numerous studies have indicated that Eda is a critical regulator of skin appendage placodes (reviewed in 7,19). In mice, the pelage hair follicles develop in three separate waves that are thought to give rise to different types of hair shafts (20,21). The first hair placodes appear at embryonic day 14 (E14), whereas the second and third wave of placode formation takes place at around E16 and E18, respectively. For clarity, these are called primary, secondary and tertiary hair placodes throughout the rest of the manuscript. A hallmark of a defect in the Eda pathway is the absence of primary hair follicles that give rise to the long guard hairs protruding above the mouse coat. However, the development of secondary and tertiary hair follicles (giving rise to awl, auchene, and zig zag hairs in wild-type mice) is initiated normally, although these follicles produce abnormal, awl-like hair shafts (19,22–25).

Analysis of reporter mice that express β-galactosidase under an NF-κB responsive promoter has revealed NF-κB activity in a number of skin appendages including those that are not severely affected in *Eda*−/− mice such as mammary glands and mystacial vibrissae (26,27). Moreover, mice overexpressing Eda-A1 in developing ectoderm (K14-Eda-A1 mice) are characterized by ectopic and enlarged hair, vibrissae, tooth and mammary placodes which develop into mature organs (28,29) (Mikkola, unpublished data). Together, these findings suggest that Eda might act redundantly with another (TNF) pathway to activate NF-κB. An obvious candidate is Troy (also known as TNFRSF19 or Taj), a TNF receptor homologous to Edar and Xedar (30–32). Expression patterns of Edar and Troy are strikingly similar with overlapping expression in the developing tooth, vibrissae and mammary gland (33), as well as in secondary and tertiary hair follicles (24,32). The exact molecular details of Troy signalling have remained elusive. A recent report suggests that Troy is activated by the TNF family ligand lymphotxin-α (LTα) (34), whereas previous studies have not revealed specific interactions between Troy and any of the TNF family members (35). In mice, loss of *Troy* does not cause any gross abnormalities (36) and a recent study suggests that an inactivating mutation in *Troy* has no effect on hair development (34).

Here we report the phenotypes of *Troy*−/− mice and *Eda*;*Troy* double mutants. *Troy* null mice displayed no apparent defects in skin appendages. However, analysis of compound *Eda* and *Troy* double mutants unveiled a critical role for Troy in hair follicle development. In addition to the absence of primary hair follicles, *Eda*;*Troy* double mutant mice lacked also secondary hair follicles. Moreover, the double mutant mice had focal alopecia in the mid-crown due to the absence of all hair follicles. The analysis of NF-κB activity in transgenic reporter mice revealed prominent reporter expression in developing ectodermal organs which was lost in almost all skin appendages including secondary hair placodes in *Eda* mutant embryos. These data indicate that *Eda* is the principal activator of NF-κB in embryonic ectoderm. Unexpectedly, our results suggest that *Eda* signals also via NF-κB independent pathway(s) *in vivo*.

RESULTS

**Troy null mice have no defects in skin appendages**

*Troy* null mice were generated by using a gene trap insertional system. The insertion was located in the first intron of the *Troy* gene (Fig. 1A). The absence of the *Troy* mRNA in homozygous mutants was confirmed with RT–PCR (Fig. 1B). The mice were viable and fertile and displayed no apparent defects in ectodermal organs. Female mice were capable of nursing normally and had normal number of nipples. No developmental abnormalities of the mammary glands were revealed when assessed by whole mount *in situ* hybridization with *Lef1*, a marker for mammary buds, in E12 embryos (Fig. 2A). Tooth number and shape and the composition of the hair types in dorsal skin did not differ from wild-type mice (Fig. 2B and C). No apparent changes in the fine structure of the hairs were detected (Fig. 2D and data not shown) and sweating ability was normal (data not shown).

**Troy and Eda double-deficient mice resemble *Eda* single mutants**

In order to study the possible redundancy of Troy and Eda pathways, we generated mice that were null for both genes. From a cross between *Eda*/*Y*;*Troy*+/*−* male and *Eda*/*X*;*Troy*+/*−* female mice, we obtained an anticipated ratio of double null mice (see Supplementary Material, Table S1) indicating a lack of embryonic lethality. The double mutants lived to adulthood and were fertile. No gross differences in body weight between the double mutants and their *Eda*−/− litter mates were observed. Female mice had a normal number of developing mammary placodes (Fig. 2A) and the wild-type number of nipples and were capable of nursing their offspring.

Mice have one incisor and three molars in each quarter of their jaw. In *Eda* mutants, the molar teeth have a reduced number of cusps, and incisors and third molars are missing with a low penetrance (37). The tooth patterns of double knockouts resembled those of *Eda*−/− mice (Fig. 2B). Mouse fur consists of four different types of hairs, guard hairs, awls, auchenes and zigzags. The fur of compound mutants consisted of only one hair type, abnormal awls, similar to *Eda*−/− mice (Fig. 2C and D). *Eda*−/− mice lack sweat glands. We performed sweat tests on *Eda*;*Troy* compound mutants (*n* = 6), and no sweat was produced, as expected (data not shown).

*Troy* has been shown to be expressed in secondary hair follicles (32) but the primary hair placodes at E14 were reported to be void of *Troy* transcripts (24). The obvious conclusion was that the difference in primary and secondary follicle
development in Eda−/− mice could be explained by redundancy of Edar and Troy signalling in secondary follicles that would be lacking in primary hair follicles due to an absence of Troy expression (24). We performed whole mount in situ hybridization with a Troy probe in E14 embryos and noticed consistent, albeit weak, Troy expression in hair placodes (Fig. 2E and F). We conclude that the lack of redundancy in primary hair placodes is most likely not due to an absence of Troy expression.

Secondary hair follicles are absent in compound Eda;Troy mutants

The appearance of body hairs seemed delayed in the double mutant pups when compared with Eda single mutants (data not shown). In principle, this could be either due to abnormal growth of the hair shaft or to lack of additional waves of hair placode formation. Therefore, we analysed the formation of hair follicles in Eda−/−;Troy−/− mutants and compared it with their Eda−/−;Troy+/− litter mates. Since it is well established that Eda null mice lack primary hair follicles (7), we focused on stages between E16 to birth. At E16 and E17, secondary (awl) hair follicles were evident in Eda−/−;Troy+/− deficient dorsal skin as previously shown for Eda−/− skin (23,38) (Fig. 3A and data not shown). At these stages, no hair follicles were initiated in the double null littermates (Fig. 3B and data not shown). Accordingly, the expression of Sonic hedgehog (Shh) was readily observed in secondary hair buds of Eda−/−;Troy+/− embryos, whereas no expression was detectable in double null mutants at E17 (Fig. 3C and D). At E18, secondary hair follicles had further invaginated into the mesenchyme in Eda−/−;Troy+/− embryos. In addition, we observed nascent hair placodes, apparently representing tertiary follicles, in both genotypes (Fig. 3E and F), which had progressed into hair buds a day later (Fig. 3G and H). These data clearly demonstrate failure in the formation of secondary hair follicles in the absence of both Eda and Troy signalling and reveal, for the first time, an important function for Troy in hair follicle development.

As both Eda-A1 and Eda-A2 isoforms of ectodysplasin are missing in Eda mutant mice, Troy could be redundant with Edar, Xedar or both. To address this question, we tested the ability of recombinant Eda-A1 and Eda-A2 to rescue secondary hair follicles in double-deficient skin explants in vitro. The experiment was initiated at E15.5, i.e. at the time when the very first molecular signs of secondary hair follicle formation can be detected (39) (see also Fig. 6C). Dorsal skin of Eda−/−;Troy−/− embryos was isolated and separated into two halves along the dorsal midline. One half was cultured in the presence of recombinant Fc-Eda-A1 or Fc-Eda-A2 (40) and the other half was used as a control. Fc-Eda-A1 restored hair placodes in the double mutant skin after 24 h of culture, whereas Fc-Eda-A2 had no effect (Fig. 3I–K), even when provided at high concentrations (up to 1 µg/ml) (data not shown). Accordingly, hair buds were observed in histological sections of Fc-Eda-A1 treated skin explants after 48 h of culture but not in Fc-Eda-A2 treated samples (Fig. 3L–N). These data suggest that Eda-A1/Edar rather than Eda-A2/Xedar functions redundantly with Troy in secondary hair placode formation.
Mid-cranial hairs are missing in compound Eda;Troy mutants

*Eda* single mutants exhibited alopecia behind their ears, as previously reported, but *Eda*;*Troy* double mutants also displayed a bald patch at the top of the vertex (Fig. 4A). There was variation in the severity of the cranial phenotype, with the size of the hairless region ranging from a barely detectable patch to a large hairless area extending into the neck where it occasionally laterally widened (Fig. 4A and data not shown). This alopecia was always symmetrical. In most severely affected mice, there was typically a sharp boundary between the naked and hairy region at the shoulder level (Fig. 4B). None of the double mutants exhibited bare patches dorsal to the shoulder line and the patch never extended anteriorly below the eyes. Alopecia was not sex-dependent as both genders showed the phenotype. Inflammation was not associated with the alopecia, assessed either by external examination or by histological analysis at different time points (Fig. 4H and data not shown).

To analyse the possibility that the lack of cranial hair was progressive or associated with hair cycling, we carefully followed the appearance of cranial hair shafts in newborn pups. Local alopecia in the cranium was evident already at the time the first hairs emerged and did not progress with time (Fig. 4C–E) indicating that the defect was neither age-dependent nor associated with hair cycling.

The bulk of the studies on hair development have been performed on dorsal skin, and little has been reported on cranial hair development. Previous studies have indicated that the hair type composition is roughly similar throughout the entire body (41,42). We analysed the hair composition in the middle cranium and in upper dorsal skin in wild-type and *Troy* null mice (Supplementary Material, Table S2). All hair types were found in the mid-scalp, although the proportion of auchene hairs was slightly lower in the cranial skin compared with the dorsal skin. This difference was seen both in wild-type and *Troy* null mice. In *Eda* null mice, the cranial hair

---

**Figure 2.** Teeth, mammary glands, and hair shaft of *Troy* null mice resembled wild-type, and *Eda*;*Troy* double mutant mice *Eda* null mice. (A) Mammary placodes were present at E12 in *Troy* null, *Eda* null and *Eda*;*Troy* double mutant embryos as indicated by the expression of *Lef1*. Comparison of the molar teeth [(B) top view, anterior is to the left], plucked hairs from upper dorsum (C) and fine structure of hairs (D) of adult wild-type, *Troy* null, *Eda* null and *Eda*;*Troy* double mutant mice. g, guard hair; au, auchene; zz, zig zag. (E) *Troy* expression in E14 hair placodes, (F) is a close-up of (E).
consisted entirely of awl-like hairs, similar to Eda−/− hairs in the upper dorsum.

Analysis of cranial hair follicle development during embryogenesis was performed to compare Eda−/−;Troy−/− and their Eda−/−;Troy+/− litter mates at E17 (A and B), E18 (E and F) and E19 (G and H). Note the absence of hair follicles at E17 in double mutants (B and D) and the appearance of newly formed tertiary placodes at E18 that bud into the mesenchyme by E19 both in Eda−/−;Troy+/− (E and G) and Eda−/−;Troy−/− (F and H) embryos. Shh was expressed in secondary hair buds at E17 in Eda−/−;Troy+/− (C) but was absent in the double mutants (D). Secondary buds are indicated by blue arrow head and tertiary placodes by green arrows in (E and F). (I–N) Secondary hair bud formation can be restored by application of recombinant Eda-A1 but not with Eda-A2. E15.5 skin explants of Eda;Troy double mutants were cultured for 24 h (I–K) or 48 h (L–N) in the presence of control medium (I and L) or 0.1 μg/ml of Fc-Eda-A1 (J and M) or Fc-Eda-A2 (K and N). Explants were analysed for hair follicle development by whole-mount in situ hybridization with placode marker β-catenin (I–K) or sectioned and stained with haematoxylin and eosin (L–N). Arrows indicate hair buds in (M).

Eda is the main activator of NF-κB activity during ectodermal organ development

To assess whether the dearth of additional skin appendage defects in compound Eda;Troy mutants could be due to lack of redundancy at the level of NF-κB activation, we compared NF-κB activity during ectodermal organogenesis in wild-type and Eda null embryos. Transgenic NF-κB reporter mice that express β-galactosidase under an NF-κB responsive element provide a sensitive readout of NF-κB activity in vitro and in vivo (26), and widespread NF-κB activation in the epithelium of skin appendages had already been observed (27). However, as no detailed spatiotemporal analyses of the NF-κB activity during ectodermal organogenesis has been reported, we first
explored reporter activity in developing mammary glands, hair follicles and teeth in wild-type embryos at E11 to E18.

Prominent LacZ expression was detected in mammary placodes and buds and during advancing mammary development (Fig. 5A and data not shown), as well as in developing sensory and mystacial vibrissae throughout embryogenesis (Fig. 5C and data not shown). Reporter activity was also evident in dental placodes at E12, in the bud stage teeth at E13, in the enamel knot region of the molars at E14–E15 as well as in the epithelium of bell stage teeth at E16 and at E18 (Fig. 5E and G and data not shown). Intriguingly, NF-κB activity was abolished in developing mammary glands, vibrissae or teeth in Eda−/− background at E11–E18 (Fig. 5 and data not shown). LacZ expression was also lost in a number of other epithelial structures in Eda null background, such as sub-mandibular glands (SMG), lacrimal glands, eye lashes, eye lids and in circumvallate papillae, although some residual activity was detectable in SMG at later stages of development (E17–E18) (Fig. 5 and data not shown). These results strongly suggest that during the early stages of ectodermal organ development the activation of NF-κB is solely dependent on Eda signalling and not on another NF-κB activator, such as Troy.

Recently, an independent transgenic reporter revealed the presence of NF-κB transcriptional activity in primary as well as in all later-developing hair buds (24). Likewise, we noticed LacZ reporter expression also in wild-type primary hair placodes (Fig. 6A) and in secondary placodes starting at E15.5, although the reporter activity strongly declined in all hair follicles at later stages (Fig. 6C and E and data not shown). As expected, no reporter activity was detected at the time of primary placode formation in Eda null embryos (Fig. 6B). Notably, secondary hair placodes/buds of Eda−/− embryos were also void of NF-κB reporter expression at E15.5–E17 when analysed by staining of whole embryos or isolated skin (Fig. 6D and F and data not shown). Taken together, our findings (Figs 3 and 6) suggest...
that Eda and Troy function redundantly in secondary hair placode formation but their action is transmitted intracellularly by pathways other than NF-κB. In principle, Edar and Troy could activate the same signalling cascade or two different pathways that act in parallel to regulate formation of secondary hair placodes. To our knowledge, this is the first demonstration of the in vivo relevance of an NF-κB independent pathway downstream of Eda.

**DISCUSSION**

**Troy is required for secondary hair follicle initiation**

Analysis of compound Eda;Troy-deficient mice revealed an important role for Troy in hair follicle development. Data presented in this report indicate that Troy acts redundantly with Eda pathway in secondary (awl) hair placode formation, as well as in all non-primary hair follicles in the head region. Thus, the characteristics of Eda;Troy double mutant mice represent a unique mouse phenotype that has not been previously described. Our in vitro rescue experiments suggested functional overlap between Edar and Troy signalling pathways rather than Xedar and Troy. Unfortunately, there are no specific molecular markers for primary and secondary hair buds. Therefore, it is in principle possible that the ‘rescued’ follicles (Fig. 3) were delayed primary follicles. However, based on previous results (9,28) and those reported here, we find it likely that Edar rather than Xedar is redundant with Troy. This conclusion is supported by the report showing the presence of Eda-A1 but not Eda-A2
protein in developing skin at the time of secondary hair placode formation (12).

**Mid-cranial hair follicles are regulated differentially**

Simultaneous loss of Eda and Troy signalling caused sex- and age-independent alopecia in the vertex. Alopecia or hair loss is a much-studied area of research due to its clinical significance. Most alopecias examined in mouse models result from defects either in hair shaft formation or hair cycling but the absence of hair may also be caused by failure in prenatal hair follicle formation (43). Few animal models display bald patches on middle of the scalp but dogs deficient in Eda are completely hairless on the forehead (44,45). Interestingly, keratin-1 promoter driven overexpression of Shh, a target of Edar (46), causes a bald patch in a location similar to that observed in Eda;Troy compound mutants (47), and mice with conditionally ablated APC, a negative regulator of Wnt pathway, are also characterized by a bare forehead despite displaying continuous ectopic hair follicle morphogenesis elsewhere (48). Although it is not clear whether these phenotypes are consequences of aberrant hair shaft formation or defective placode induction, these examples suggest that the cranial region is particularly sensitive to perturbations in key pathways regulating hair follicle development.

**Eda and Troy signal transduction pathways**

Several studies have pinpointed the activation of NF-κB as an important outcome of Edar signalling. However, this evidence has either been obtained from *in vitro* studies (12–14) or deduced from similarities with mice and humans mutant or transgenic for components of the NF-κB pathway (15–18). Here we show, given any potential limitations of the reporter assay, that NF-κB activity is strictly dependent on ectodysplasin during early developmental stages of skin appendages such as teeth, mammary glands and vibrissae. In line with our results, Schmidt-Ullrich *et al.* (24) have reported, using an independent NF-κB reporter construct, loss of NF-κB signalling in primary hair placodes of Eda and Edar mutant embryos, with some residual activity found in nascent hair buds (apparently tertiary follicles) in newborn mice. Together, these data indicate that Eda signalling is solely responsible for the NF-κB activation observed in primary and secondary hair follicles. Our results imply that in most skin appendages Troy is unable to activate NF-κB. This could either be an intrinsic feature of Troy or reflect the absence of a necessary component of the Troy signal transduction pathway.

The intracellular components of the Eda signalling pathway have been elucidated to a certain extent. An Edar-specific adapter molecule Edaradd is essential to bridge Edar to further downstream molecules such as Traf5 (which are common mediators in TNFR superfamily signalling), Tab2

![Figure 6. NF-κB reporter expression in developing primary and secondary hair follicles is dependent on Eda. (A) NF-κB reporter is expressed in nascent primary hair placodes in wild-type mice at E14 but not in Eda null mice (B). The larger primary hair buds (red arrow) as well as the smaller secondary hair placodes (blue arrow head) were positive for NF-κB reporter activity in wild-type embryos at E15.5 (C) but not in Eda-deficient embryos, although staining in blood vessels was readily detectable (D). Faint NF-κB reporter activity could also be detected in hair follicles of wild-type embryos at E17 (E) but not in Eda mutant embryos (F).](https://academic.oup.com/hmg/article-abstract/17/21/3380/2385855)
and Tak1, eventually leading to IKK activation (49–51). Of the six Traf molecules, Edaradd has been shown to bind Traf1, -2, -3 and -6 in vitro (49–51). Interestingly, all NF-κB activity is lost in skin appendages of Traf6−/− mice when analysed at E13 using the same reporter mice as in this study (27). In addition, Traf6 null mice display an ectodermal dysplasia phenotype highly similar to Eda and Edar-deficient mice (52,53). These findings propose a model where Eda signalling results in a Traf6-dependent activation of NF-κB (Fig. 7). Interestingly, there are few skin appendage-specific features that distinguish Traf6−/− mice from Eda−/− mice; the most prominent being the absence of hair follicles until E18 (52). As this phenotype is shared by Eda;Troy double mutants and Troy binds Traf6 in vitro (52), these results propose that in vivo, Traf6 mediates both Edar and Troy signalling (Fig. 7).

Our hypothesis that the absence of secondary hair placodes in compound Eda;Troy mutants reflects the activation of pathways other than NF-κB is based on several pieces of evidence. First, we did not observe the expression of NF-κB reporter in secondary placodes/buds in Eda−/− background. Second, although the total number of hair follicles appears to be reduced in mice with suppressed NF-κB activity (cIκBαΔN mice) when compared with Eda−/− mice, secondary hair follicles are initiated normally as in Eda−/− embryos (15). No NF-κB reporter staining can be visualized in the hair follicles of cIκBαΔN mice (E14-P0) suggesting complete inhibition of canonical NF-κB signalling (24). cIκBαΔN mice have not been reported to have local alopecia in mid-cranium, implying a critical involvement of NF-κB independent pathways downstream of Eda and Troy in the cranial phenotype.

In conclusion, our results show that Troy has a critical role in hair follicle development. Troy and Eda function redundantly in secondary hair follicles but not in other skin appendages where the expression domains of Edar and Troy overlap. Functional overlap is evident also in tertiary hair follicles in the head and neck region, suggesting that Eda has a role in the formation of all hair placodes but that it can be substituted by alternate signalling pathways (Fig. 7). One such pathway could be the Wnt/β-catenin pathway as its forced activation can compensate for the absence of Eda in primary hair placodes (54). Moreover, our data suggest that Eda-A1/Edar pathway, mediated by Traf6 (27,51), is the main activator of NF-κB in skin appendages during embryogenesis. Surprisingly, our results also indicate that NF-κB independent pathways downstream of Eda are crucial for the formation of secondary hair placodes. The identification of the other pathway(s) and the question whether NF-κB-dependent and -independent pathways execute similar tasks and regulate the same target genes during primary and secondary hair placode formation are interesting areas of future research.

MATERIALS AND METHODS

Mice and their analysis

The Eda-deficient (Tabby) mice were of B6CBACa-AW1/A-Ta strain obtained from Jackson Laboratories (Bar Harbor, ME, USA) (37). The generation and genotyping of NF-κB reporter mice has been described (26) and they were in C57Bl/6 background. NF-κBREP mice mated into Tabby background were bred into homozygosity for Eda mutation. In addition, Eda+/− mice were crossed with Eda−/−; NF-κBREP males to allow the analysis of NF-κB activity in wild-type (Eda+/Y) and Eda null (Eda−/Y or Eda−/−) embryos from the same litter. Identical results were obtained in both cases.

Troy-deficient mice were a generous gift of Wyeth Pharmaceuticals Inc. The Troy gene-trapped knockout mice were developed by Lexicon Genetics, Inc. (The Woodlands, TX, USA) using the Omnibank VICTR gene trap system. The VICTR gene trap consists of LTR arms flanking a promoterless neomycin resistance gene with a splice acceptor and a minigene composed of a phosphoglycerate kinase-1 promoter, the terminal exon of the Bruton’s tyrosine kinase gene and a splice donor sequence (55). An ES cell line with the VICTR gene was identified by conducting a BLAST search of the Omnibank database of gene-trapped ES cells. These cells were used to generate chimeric mice by blastocyst injection. The mice were maintained on the 129SvEvBrd genetic background of the ES cells. The Troy−/− mice were genotyped by PCR
analysis. Wild-type and *Troy* null alleles were distinguished using allele-specific forward primers (5′-GTGGAGAAGGAAGCTGTAGTG) and (5′-GGCTTACTTAACTCAGC), respectively, and a common reverse primer (5′-ACGGGCTTGGACATTACTTTGG) yielding a 627 bp wild-type product and ~300 bp product of the gene-trapped allele. When genotyping *Eda:Troy* double mutants, we observed in some of the progeny a larger, ~1.3 kb PCR product. This originated from a CBA strain wild-type allele from the parental *Eda*+/− strain, which was in a hybrid B6CBA background. The sequenced 1.3 kb PCR product showed an insertion within the *Troy* intronic area. The insertion shows homology to murine ribosomal protein S7 mRNA possibly representing a polymorphic pseudogene (data not shown). Transcription of a wild-type *Troy* mRNA from the CBA allele was confirmed with RT–PCR.

For RT–PCR, RNA was isolated from lung or liver samples with Trizol (Gibco). RT reaction was performed with SuperScript II (Invitrogen) with random hexameric primers for beta-actin and with 5′-CTCTGGGTCTTGTGAAACCAC oligo for *Troy*. Oligos for PCR amplification were 5′-GGAATCGATCTACCTGCTTGC and 5′-GACGAGGCCCAGAGCGAGGAG (beta-actin) and 5′-CTCTGGGTCTTGTGAAACCAC and 5′-CCCTCCTTGTGCTGTAAC (Troy). PCR was performed with Dynazyme II (Finzymes).

For hair analysis, individual hairs were plucked with forceps from the upper back and mid-cranium from adult mice and examined under a stereo or light microscope. Hair type identification, skeletal preparations of adult jaws and sweat tests were performed as described previously (28,37).

**Histology and in situ hybridization**

Staged embryos from E16 to E19/newborn and 5 and 12 days post-natal mice were collected. The tissues were fixed in 4% paraformaldehyde and taken through ethanol series and xylene into paraffin and sectioned at 7 μm for histology. Post-natal tissues were decalcified in EDTA-paraformaldehyde solution. Sections for normal histology were stained with haematoxylin and eosin. Whole-mount in situ hybridization analysis was carried out using the InsituPro robot (Intavis, Germany) as previously described (23). In situ hybridization of tissue sections was performed according to the standard protocols. *Troy*, *Shh*, *Lef1* and *β-catenin* probes were as described (23,33,46).

**X-gal staining**

For Assessment of NF-κB reporter activity in mouse tissues, whole mount X-gal staining was performed on E11–E16 whole embryos or dissected lower jaws, remaining heads and the body trunks or on isolated E16–E18 skin. In embryos older than E14, the internal organs were removed in order to enhance penetration of the staining solution. Tissues were fixed for 30 min to 2 h at 4°C in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS. When indicated, skin was dissected prior to fixation. After three washes in 2 mM MgCl2 and 0.02% NP-40 in PBS, tissues were assayed for β-galactosidase activity by incubation at room temperature in staining solution of 1 mg/ml X-gal (Promega), 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2 including 0.02–1% NP-40 in PBS for 20 h with constant agitation. Samples were washed with PBS and post-fixed in 4% paraformaldehyde in PBS.

**Tissue culture**

Back skin of E15.5 *Eda−/−* or *Troy−/−* embryos was dissected in Dulbecco’s PBS and divided into two halves along the dorsal middle. Skin-halves were grown for 1 or 2 days on Nuclepore filters at 37°C in a Trowell type of culture in DMEM supplemented with fetal calf serum, glutamine and penicillin–streptomycin as described (29). One half of the skin was cultured in the presence of indicated amounts of recombinant Fc-Eda-A1 or Fc-Eda-A2 (40) and the other half was used as a control. Cultured skin explants were processed either for whole-mount in situ hybridization (after 1 day culture) or for histology and stained with haematoxylin and eosin (after 2 days culture).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**FUNDING**

This work was supported by the Academy of Finland (M.L.M., I.T.), the Sigrid Jusélius Foundation (I.T.) and Viiikki Graduate School of Biosciences (M.P.).

**ACKNOWLEDGEMENTS**

We are grateful to Drs Andrew Long and Divya Chaudhary at Wyeth Research for the generous gift of *Troy*-deficient mice and for discussions and Pascal Schneider for recombinant Fc-Eda-A1 and Fc-Eda-A2 proteins. We thank Riikka Santalahti, Raia Savolainen, Merja Mäkinen and Heidi Kettunen for excellent technical help.

**Conflict of Interest statement.** None declared.

**REFERENCES**


