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ECTODYSPLASIN A (EDA) – EDA RECEPTOR SIGNALLING AND ITS PHARMACOLOGICAL MODULATION.

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Running title: Modulation of EDA-EDAR signalling.

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Abstract:
The TNF family ligand Ectodysplasin A (EDA) regulates the induction, morphogenesis and/or maintenance of skin-derived structures such as teeth, hair, sweat glands and several other glands. Deficiencies in the EDA - EDA receptor (EDAR) signalling pathway cause hypohidrotic ectodermal dysplasia (HED). This syndrome is characterized by the absence or malformation of several skin-derived appendages resulting in hypotrychosis, hypodontia, heat-intolerance, dry skin and dry eyes, susceptibility to airways infections and crusting of various secretions. The EDA-EDAR system is an important effector of canonical Wnt signalling in developing skin appendages. It functions by stimulating NF-κB-mediated transcription of effectors or inhibitors of the Wnt, Sonic hedgehog (SHH), Fibroblast growth factor (FGF) and Transforming growth factor beta (TGFβ) pathways that regulate interactions within or between epithelial and mesenchymal cells and tissues. In animal models of *Eda*-deficiency, soluble EDAR agonists can precisely correct clinically relevant symptoms with low side effects even at high agonist doses, indicating that efficient negative feedback signals occur in treated tissues. Hijacking of the placental antibody transport system can help deliver active molecules to developing foetuses in a timely manner. EDAR agonists may serve to treat certain forms of ectodermal dysplasia.

Ectodysplasin A and ectodermal dysplasias

Ectodermal dysplasias comprise more than 180 different heritable syndromes affecting at least two ectodermal structures such as hair, teeth, nails or exocrine glands (Visinoni et al., 2009). The most common ectodermal dysplasia, X-linked hypohidrotic ectodermal dysplasia (XLHED), is characterized by missing or sparse hair (hypotrychosis), abnormal or missing teeth (hypodontia or anodontia), reduced sweating ability (hypohidrosis), and defects of various lipid- or mucus-secreting glands. XLHED is caused by mutations of the ectodysplasin A (*EDA*) gene (Kere et al., 1996). *EDA* mutations are also associated with selective tooth agenesis (STA), an inherited condition in which teeth only are affected, with no pathologic involvement of other ectodermal appendages (Mues et al., 2010). EDA is a TNF family ligand that binds to EDA receptor (EDAR). EDAR signals via an adaptor protein, EDAR-associated protein with a death domain (EDARADD). Mutations in the *EDAR* or *EDARADD* genes cause hypohidrotic ectodermal dysplasias identical to XLHED, except for their autosomal dominant or recessive modes of transmission (Headon et al., 2001; Headon and Overbeek, 1999).

Ectodysplasin A

The human *EDA* gene was first identified by positional cloning in XLHED patients and is located on the long arm of the X chromosome (Kere et al., 1996). EDA is a 391 amino acid residues-long membrane protein with a short intracellular domain, a transmembrane domain, a stalk region of uncharacterized function, a consensus furin cleavage sequence responsible for proteolytic processing of EDA, a short positively-charged sequence required for interactions with heparan-sulfate proteoglycans, a bi-partite collagen-like domain and a 150 amino acid residues-long C-terminal TNF homology domain (THD) responsible for receptor binding (Fig. 1A). *EDA* transcripts undergo complicated splicing events giving rise to numerous EDA isoforms (up to nine in mouse keratinocytes), of which only the longest two, EDA1 and EDA2, contain the THD, interact with receptors and are known to be biologically active (Bayes et al., 1998; Hashimoto et al., 2006). EDA1 and EDA2 differ by two amino acid residues in the THD (Glu308 and Val309) as a result of differential usage of a splice donor site at the end of exon 7 (Fig. 1A) (Yan et al., 2000). EDA1 binds to EDAR, while the 2 amino acid residues shorter EDA2 specifically interacts with another TNF receptor family member, XEDAR (Yan et al., 2000). EDA1, EDA2 and their receptor binding specificity are conserved in mammals and birds (Kowalczyk-Quintas et al., 2014). *XEDAR* is a p53-induced gene with no obvious implications in ectodermal appendage development (Brosh et al., 2010; Newton et al., 2004). *EDA* mutations identified in XLHED patients touch several distinct regions of the protein that are therefore believed to be of functional importance (Schneider et al., 2001) (Fig. 1A). The first one lies at the beginning of the stalk region, but why this region is important for EDA expression or function is unknown. Interestingly, fishes that have evolved in fresh water display a dramatic reduction in their bony armour compared to ocean fishes of the same species, which is due to the selection of a low-abundance allele of *Eda* that includes, among other polymorphisms, a Leu79Pro transition at the beginning of the stalk region (Colosimo et al., 2005). The second region is the furin consensus cleavage site, indicating that EDA1 must be released to a soluble form to display activity (Fig. 1A, B). The third region is the THD. The THD forms homotrimers that can bind three individual receptors at each monomer-monomer interface (Hymowitz et al., 2003). XLHED- and STA-causing mutations in this domain interfere either with trimer formation, or with receptor binding (Mues et al., 2010; Schneider et al., 2001). The fourth region is the collagen domain that serves to keep two or more EDA1 trimers in close proximity within the same molecule and therefore

potentiate its ability to stimulate EDAR signalling (Schneider et al., 2001; Swee et al., 2009) (Fig. 1A,B). Finally, the proteoglycan-binding domain of EDA, which is mostly coded by the very short exon 3, may restrict EDA diffusion in tissues once it is released in a soluble form (Swee et al., 2009) (Fig. 1B). Mutations in the proteoglycan-binding region have not been reported in XLHED patients, either because this interaction is not essential for the function of EDA, or because disruption of the interaction can be achieved neither with a single point mutation nor with exon 3 deletion, which would induce a frame shift.

EDAR

EDAR is a typical TNF receptor family member with a signal peptide, three cystein-rich domains (CRDs), a transmembrane domain and an intracellular region comprising a so-called "death domain" (Headon and Overbeek, 1999) (Fig. 1A). Most mutations in patients with autosomal HED are located within CRD2, which is involved in ligand binding, or within the death domain, which is involved in signal transmission (Fig. 1A). A function activating polymorphism (Val370Ala) found in the death domain of EDAR in East Asian and Native American populations is associated with a thicker hair phenotype (Mou et al., 2008; Sabeti et al., 2007). In the death receptors Fas and TRAILR2, the death domain recruits Fas-associated protein with a death domain (FADD), that itself recruits and activates cystein proteases of the caspase family to execute apoptotic cell death. EDAR does not interact with FADD, but instead recruits EDARADD, another adaptor protein with a death domain (Headon et al., 2001) (Fig. 1A).

EDAR activates canonical NF-kB

Deficiencies for EDA, EDAR or for the adaptor protein EDARADD all induce HED with similar if not identical manifestations. EDARADD, unlike FADD, does not recruit caspases, but possesses consensus binding sites for TNF receptor associated factors (TRAFs), and in particular TRAF6 (Fujikawa et al., 2013; Headon et al., 2001). TRAF6 mediates the activation of the transcription factor NF-κB downstream of several TNF receptor family members (*e.g.* EDAR, RANK and CD40) or other receptors (IL1R, TLRs). Thus, *Traf6*-deficient mice do not only develop ectodermal dysplasia similar to *Edar*- or *Edaradd*-deficient mice, but also osteopetrosis, alymphoplasia -RANK signalling is essential for the generation of osteoclasts and lymph nodes-, B cell abnormalities -CD40 is important for B cell activation- and blunted signalling by IL1 receptor and Toll-like receptors (Naito et al., 2002). TRAF6 is an E3 ubiquitin ligase that generates K63-linked polyubiquitin chains to which binds NEMO, the regulatory component of the inhibitor of NF-κB kinase (IKK) complex (Chen, 2012). *NEMO* is on the X chromosome. Although complete *NEMO*-deficiency causes a severe and lethal inflammatory skin phenotype in human males, hypomorphic *NEMO* mutations are viable and cause HED with immunodeficiency, sometimes complicated by osteopetrosis for reasons similar to those explained above for *Traf6*-deficiency (Smahi et al., 2002; Zonana et al., 2000). The crucial implication of NF-κB downstream of EDAR is demonstrated by the phenotype of IκBαΔN transgenic mice that express a non-phosphorylable, and therefore non-degradable form of a NF-κB inhibitor. These mice suffer from HED, osteopetrosis and immunodeficiency (Schmidt-Ullrich et al., 2001). In embryonic skin of wild type mice, NF-κB is activated in primary hair placodes (Schmidt-Ullrich et al., 2006). In *Eda*-deficient skin, there are no placodes and there is no NF-κB activation. Addition of ectopic recombinant EDA restores placodes and NF-kB signals in *Eda*-deficient skin, but not in *IκBαΔN* transgenic skin (Schmidt-Ullrich et al., 2006). At least for the formation of primary hair placodes, NF-κB activation downstream of EDAR is not only

required, but apparently also sufficient as placodes can be formed with other NF-kB stimulators such as TNF or LPS (Schmidt-Ullrich et al., 2006) (Table 1) (Fig. 2). Table 1 is a compilation of phenotypes observed upon genetic modifications of genes related to processes also regulated by EDAR-signalling. Figure 2 is a partial, naïve and certainly incorrect view of how these gene products may interact with each other in the early phases of hair and tooth development.

The EDA - EDAR pathway is an important effector of Wnt signalling, and in turn promotes further Wnt signalling

Primary hairs do not form in *Edar*-deficient or in Wnt signalling-deficient (deletion of *β-catenin*) mice (Headon and Overbeek, 1999; Huelsken et al., 2001). Early studies in conditional *β-catenin* knock-out mice, where *β-catenin* was deleted in epithelial cells with a Keratin14 promoter-driven Cre recombinase, concluded that Wnt signalling might be downstream of EDAR, as *Edar* was the only placode marker still expressed in *β-catenin*-deficient E15.5 skin (Huelsken et al., 2001). Also, *Edar* expression was unaffected in *Lef-1*-null skin and tooth buds (Laurikkala et al., 2001; Laurikkala et al., 2002). This conclusion that *Edar* was not a Wnt-responsive gene was subsequently revised when *β-catenin* was deleted with another Keratin14 promoter-driven *Cre* recombinase with more precocious expression. Indeed, *Edar* expression and NF-κB activation in E14.5 skin was absolutely dependent on β-catenin activity in the epithelium, and *Edar* was characterized as a direct Wnt target gene (Zhang et al., 2009). Likewise, initial uniform expression of *Eda* is also Wnt-dependent (Durmowicz et al., 2002; Laurikkala et al., 2001; Zhang et al., 2009). Although Wnt signalling acts upstream of EDAR in primary hair placodes, maintenance of β-catenin activity in primary placodes at a later stage requires EDAR activity (Zhang et al., 2009). EDAR signalling induces expression of *Wnt10b*, a direct NF-κB target gene, which is a good candidate to mediate the second round of β-catenin signalling in placodes (Zhang et al., 2009). Interestingly, forced EDAR-signalling (by expression of a constitutively active *LMP-EDAR* fusion receptor) in primary hair placodes in the context of Wnt inhibition (by expression of the Wnt inhibitor DKK1) failed to rescue expression of EDAR target genes such as *Shh*, indicating that expression of Wnt-dependent genes other than *Eda* and *Edar* is required for successful EDAR signalling (Zhang et al., 2009) (Fig. 2).

Development of skin-derived appendages

Skin-derived appendages, such as hair, teeth, and mammary glands have different function and structure but share common developmental aspects. The first step of development is the formation of a thickening of the epithelial layer, the placodes, accompanied by the condensation of the underlying mesenchyme. Placodes are embryonic signalling centres that express signalling molecule such as Wnt, FGF, TGFβ, Hedgehog and TNF family proteins (Laurikkala et al., 2002; Mikkola, 2007). In the next step of development, placodes invaginate into the mesenchyme to form buds that further grow and develop in organ-specific manners. Interactions between the epidermal and underlying dermal cells and tissues are common and recurrent themes of skin-derived organ development.

Development of hair

In mice, hair development occurs in three distinct and successive waves giving rise, respectively, to guard, awl and auchene, and zigzag hair types. The first wave of hair placodes is visible at E13.5, and the second and third are initiated at E16 and E18 (Schmidt-Ullrich and Paus, 2005). The placode is formed by a clustering of epidermal keratinocytes in response to a signal from the

dermis that could be Wnt or a Wnt inducer. Placodes express inducing molecules to re-enforce signalling, and inhibitors to prevent differentiation at the periphery of placodes until a regular array of hair follicles is established (Millar, 2002; Schmidt-Ullrich and Paus, 2005). Each placode induces underlying mesenchymal cells to aggregate and form the dermal condensate (Millar, 2002). This is followed by proliferation and down growth of epithelial cells into the dermis until they surround the dermal condensate that becomes the dermal papilla. Lateral communication between the dermal papilla and the follicular epithelium induces proliferation of epithelial matrix cells that give rise to different hair follicle lineages forming first the different cell types of the inner root sheath (Henley's layer, Huxley's layer and cuticle) and then those of the mature hair shaft (cuticle, medulla and cortex) (Millar et al., 1999). The inner root sheath is surrounded by the companion cell layer and by the outer root sheath that is contiguous and similar to the epidermal basal layer.

Development of teeth

In teeth, the odontogenic signal originates form the epithelium at E11. At E12, placode cells proliferate into the underlying mesenchyme to form a bud. At E13.5 the mesenchyme condenses around the bud, expresses signalling molecules and transcription factors and instructs the epithelium to form the primary enamel knot at the tip of the bud. The primary enamel knot is a non-proliferating signalling centre important to stimulate cell proliferation in surrounding tissues. The primary enamel knot thus regulates folding of the tooth epithelium in a cap-shaped structure at E14.5 (Dassule et al., 2000; Jernvall and Thesleff, 2000). At the bell stage (E16), the condensed mesenchyme -now called the dental papilla- is completely enclosed by the invaginating tooth epithelium. Enamel knots are transient structures that disappear by apoptosis at the end of the cap stage (Jernvall and Thesleff, 2000). Incisor tooth germs have a single enamel knot, whereas molars additionally form secondary enamel knots at the bell stage to fold the enamel epithelium again and form multi-cuspids teeth. The final tooth shape is defined by the folding of the inner enamel epithelium and by the growth of the dental papilla (Caton and Tucker, 2009).

Wnt and Wnt inhibitors

Wnt/β-catenin signalling is crucial for the development of ectodermal appendages at early and later stages of differentiation (Andl et al., 2002; Huelsken et al., 2001; Liu et al., 2008; Millar, 2002; Schmidt-Ullrich and Paus, 2005; van Genderen et al., 1994). The canonical Wnt pathway requires β-catenin. When Wnt is absent, β-catenin is eliminated by the action of the AXIN complex, which triggers β-catenin to proteasomal degradation. When Wnt is present, it binds to Frizzled receptor and to LRP5/6 co-receptors, which recruit Dishevelled (DVL), resulting in LRP5/6 phosphorylation and recruitment of the AXIN complex. This inhibits β-catenin degradation. Accumulated β-catenin translocates to the nucleus where, in complex with T cell factor/lymphoid enhancer factor (LEF/TCF), it activates expression of Wnt target gene (MacDonald et al., 2009). Negative regulators of Wnt signalling include DKKs (Andl et al., 2002) and Sostdc1 (also known as Ectodin or Wise) (Ahn et al., 2010; Narhi et al., 2012). Both negatively regulate LRP5/6 co-receptors and therefore Wnt signalling, either directly (Sostdc1) (Itasaki et al., 2003; Li et al., 2005; Lintern et al., 2009) or additionally via engagement of Kremens (DKKs) (Wang et al., 2008) (Fig.2). *Dkks* are Wnt- and NF-κB-responsive (Andl et al., 2002; Fliniaux et al., 2008; Zhang et al., 2009), whereas *Sostdc1* is activated by BMPR signalling (Laurikkala et al., 2003; Mou et al., 2006). Sostdc1 is also a BMP antagonist

(Laurikkala et al., 2003), but the phenotype of *Sostdc1*-deficient mice that is characterized by enlarged enamel knots, additional teeth, enlarged mammary buds, enlarged hair placodes and ectopic whiskers (Ahn et al., 2010; Kassai et al., 2005; Narhi et al., 2012), is probably due to an upregulation of the Wnt signalling pathway (Ahn et al., 2010; Narhi et al., 2012). Endogenous expression of *Dkk1* in the dermal condensate is suppressed by DKK1 overexpression, suggesting that it is a direct target of Wnt signalling involved in a negative feedback loop (Andl et al., 2002).

TGFβ family members BMP, Activin, and their inhibitors

BMPs are known as placode inhibitors (Botchkarev et al., 1999), but also provide positive signals for the differentiation of inner root sheath cells (Kobielak et al., 2003), generation and decay of enamel knots (Jernvall et al., 1998) or differentiation of ameloblasts (Wang et al., 2004). The action of BMP is restricted by expression of a range of antagonists: Sostdc1 (Laurikkala et al., 2003), CTGF (also known as CCN2) (Mou et al., 2006; Pummila et al., 2007), Noggin (Botchkarev et al., 1999) and Follistatin (Wang et al., 2004). Cell responses to BMP are often regulated by gradients. In the hair follicle, *Bmpr1a* ablation prevents differentiation of matrix cells to inner root sheath cells, and therefore prevents hair shaft formation (Andl et al., 2004; Kobielak et al., 2003). A model for the differentiation of matrix cells to inner root sheath cells takes into account the seemingly contradictory observations that BMPR1a signalling inhibits *Lef-1* expression, yet is required for β-catenin/LEF-1 activation (Kobielak et al., 2003). In the hair follicle, the dermal papilla produces Noggin that inhibits BMP signalling in the nearby proliferating epithelial matrix cells. Inhibition of BMP signalling induces *Lef1* and *Bmp4* expression in matrix cells, but β-catenin/LEF-1 activation does not take place in the absence of BMPR1a signalling, possibly due to down-regulated expression of Wnt receptors or to an other cause. Absence of Wnt signalling thus prevents differentiation of proliferating matrix cells. As proliferating cells move upwards and away from the source of Noggin, they start responding to their own BMP4 to become Wnt-responsive. Despite *Lef-1* downregulation, stabilized LEF-1 protein is sufficient to ensure efficient β-catenin/LEF-1 activation and expression of Wnt-responsive genes (*e.g.* hair keratins, *Foxn1, Msx1*, *Msx2* and *GATA3*) involved in the differentiation of matrix cells to inner root sheath cells, which themselves will give rise to hair shaft cells (Kobielak et al., 2003).
ActivinβA is a TGFβ family member involved in tooth and whiskers formation (Ferguson et al., 1998; Jhaveri et al., 1998; Matzuk et al., 1995). ActivinβA, its receptor ACTRII and its effector SMAD2 are all essential for the development of incisors and mandibular molars (Ferguson et al., 1998; Ferguson et al., 2001). Follistatin inhibits ActivinβA (Jhaveri et al., 1998; Matzuk et al., 1995; Wang et al., 2004) (Fig. 2).

Sonic hedgehog

Sonic hedgehog (SHH), a member of the Hedgehog family, is a secreted protein important for organizing dermal cells into a dermal condensate (Chiang et al., 1999; St-Jacques et al., 1998). At a subsequent developmental stage, it induces proliferation of epithelial cells (Dassule et al., 2000; Hardcastle et al., 1998). Binding of SHH to Patched releases the inhibition imposed on Smoothened that in turn can convert the Gli transcription factors from transcriptional repressors to transcriptional activators (Fig. 2).

FGF

FGFs promote cell growth and proliferation. The direct Wnt target genes FGF4 and FGF8 produced in the epithelium are essential for tooth development (Kratochwil et al., 2002; Wang et al., 2009). Interestingly, FGF8 controls expression of *activinβA*, that is essential for mandibular (but not maxillary) molar development, and of the homeobox genes *Dlx1* and *Dlx2* that are required for maxillary (but not mandibular) molar development (Ferguson et al., 2001) (and references therein). In chickens, the *scaleless* mutation disrupts the *Fgf20* gene and results in naked, featherless and scaleless birds (Wells et al., 2012). In mice, *Fgf20* is required for the formation of dermal condensations in primary and most secondary developing hair follicles, resulting in mice with no guard, reduced awl and auchene, and normal zigzag hair, in addition to moderately abnormal teeth (Haara et al., 2012; Huh et al., 2013). MAPK activation by FGF receptors is inhibited by Sprouty proteins, which are commonly induced by FGF action (Fig. 2).

Molecular targets of EDAR signalling

EDA and EDAR are important for the development of some but not all placodes (Laurikkala et al., 2002; Schmidt-Ullrich et al., 2006). EDA plays a role for stabilizing primary hair placodes, more that initiating them, as suggested by the presence of transient hair pre-placodes at E14 in *Eda*-deficient skin (Mou et al., 2006; Schmidt-Ullrich et al., 2006). Even when EDAR signalling is dispensable for placode formation, it often regulates morphology of the appendage or gland (Hammerschmidt and Schlake, 2007; Pispa et al., 1999; Schmidt-Ullrich et al., 2006; Voutilainen et al., 2012; Wells et al., 2010). EDAR signalling potentially interacts with all major morphogenesis pathways (Fig. 2). EDAR regulates expression of *Edar* itself (Mou et al., 2006), *Wnt10b* (Zhang et al., 2009), *Dkk4* (Fliniaux et al., 2008), *Shh* (Pummila et al., 2007), *Ctgf* (Mou et al., 2006; Pummila et al., 2007), *Follistatin* (Pummila et al., 2007) and *Fgf20* (Haara et al., 2012; Huh et al., 2013). BMP4 strongly inhibits *Edar* expression in embryonic skin and could explain its negative role on primary placode formation (Mou et al., 2006). The induction of BMP inhibitors may locally protect the placode from BMP action, while the immediately surrounding tissue down-regulates *Edar* (Mou et al., 2006). Production of Wnt inhibitors may also contribute to downregulate *Edar* and other relevant genes at the periphery of placodes. The absence of *Shh* and *Fgf20* expression in primary hair pre-placodes of *Eda*-deficient skin, and reduced production in *Eda*-deficient enamel knots certainly explains much of the absence of guard hair and of the molar defects of *Eda*-deficient mice (Haara et al., 2012; Huh et al., 2013; Pispa et al., 1999; Pummila et al., 2007). In addition, the branching defect observed in salivary glands of *Eda*-deficient mice can be rescued in cultured organ explants with ectopic SHH (Wells et al., 2010), supporting a relevant role for SHH downstream of EDAR.

Mouse and dog models of X-linked hypohidrotic ectodermal dysplasia

In the mouse, loss-of-function mutations of *Eda* cause the Tabby phenotype, which is the mouse counterpart of XLHED (Srivastava et al., 1996; Srivastava et al., 1997). Tabby mice have a single abnormal awl-like hair type, no hair on the tail, lack the fine hair behind the ears but have hair on the ear skin. They lack sweat glands, mebomian glands, tracheal glands and present more or less marked abnormalities in sebaceous glands, mammary glands and salivary glands (Gruneberg, 1971).

A dog model of XLHED is also available (Casal et al., 1997), in which a mutation in a splice site of EDA prevents protein expression (Casal et al., 2005). Affected dogs lack sweat glands, have bald patches around the body and lack secondary hair. They present abnormal or missing teeth, suffer from dry eyes and from recurrent airways infections due to abnormal mucus secretion in

bronchi and trachea (Casal et al., 1997; Casal et al., 2005). Thus, HED dogs recapitulate several of the clinically relevant defects of XLHED patients.

Pharmacological activators of EDAR

Fc-EDA1 is an EDAR agonist. It is a fusion protein between the effector portion (Fc fragment) of human IgG1 and the C-terminal, THD domain of EDA1 (Gaide and Schneider, 2003). As the Fc is a dimer and the THD is a trimer, the fusion protein mainly assembles as hexamers containing three Fc and two THD (Fig. 1C). The protein lacks the difficult-to-express collagen domain of EDA, whose aggregating function is mimicked by fusion with the Fc. Fc-EDA1 also lacks the proteoglycan-binding portion that prevents access of recombinant EDA to relevant tissues *in vivo* upon intra-peritoneal administration (Swee et al., 2009). The Fc portion of Fc-EDA1 is also important to a) enhance half-life of the protein *in vivo*, an effect that is well-known to be mediated by binding to neonatal Fc receptors (FcRn), and b) allow transport from the maternal circulation to the circulation of the foetus via the trans-placental antibody transport system, which allows exposure of foetuses to the protein *in utero*. Fc-EDA1 can substitute for endogenous EDA1 to correct many phenotypic abnormalities of *Eda*-deficient mice or dogs, including permanent teeth in mice and the secondary dentition in dogs (Casal et al., 2007; Gaide and Schneider, 2003; Mauldin et al., 2009). In dogs, post-natal administration of Fc-EDA1 had several direct clinical benefits: it permanently abolished the dry eye phenotype (reverted dogs no longer needed the chronic eye drop treatment required by affected dogs) and improved mucociliary clearance in the airways, a feature that correlated with a drastic reduction of airways infections and courses of antibiotic treatments (Casal et al., 2007). Several anti-EDAR antibodies raised in *Edar*-deficient mice display *in vivo* activities similar to that of Fc-EDA1 when administered to *Eda*-deficient mice or dogs (Kowalczyk et al., 2011). Several hallmarks of the reversion of *Eda*-deficient animals with EDAR agonists are worth mentioning. A first feature is the life-long phenotypic correction achieved by a short-course treatment, indicating that EDAR provides an inductive rather than a maintenance signal (Gaide and Schneider, 2003; Kowalczyk et al., 2011) (Fig. 3). However, hair and their associated sebaceous glands are known exceptions in which the EDA-EDAR pathway remains active and can be regulated in adult life (Cui et al., 2003; Fessing et al., 2006) (CKQ and PS, unpublished data). The effect of chronic EDAR-agonist treatment on hair and their associated sebaceous glands will be interesting to study in the future. A second important feature of treatment efficacy is its relatively narrow therapeutic window, which depends on the ability of developing tissues to respond to EDA (Gaide and Schneider, 2003). Finally, the surprising fidelity with which tooth morphology can be corrected in *Eda*-deficient animals treated with a vast excess of "artificial" soluble EDAR agonists is certainly due to the fact that EDAR expression and its ability to signal and initiate regulatory feed-forward and feed-back responses are all intact in *Eda*-deficient animals. Another example is the restoration, in mice, of tail hairs regularly arranged by groups of three, which is remarkable considering that the skin of the tail is completely naked in the absence of treatment (Gaide and Schneider, 2003; Kowalczyk et al., 2011). However, soluble agonists do not restore the characteristic morphology of zigzag hairs with three evenly spaced and well-marked kinks. These kinks find their origin in the asymmetric expression of *Eda* in matrix cells of the hair follicle, which periodically shifts from one side of the hair follicle to the other, while *Edar* is continuously expressed on both sides of the follicle (Hammerschmidt and Schlake, 2007). EDAR signalling in the follicle, as judged by *Shh* and *Saa3* expression -Saa3 is a NF-κB responsive gene- reflects the *Eda* expression pattern, suggesting that EDA acts locally (Hammerschmidt and

Schlake, 2007), possibly because the proteoglycan-binding domain restricts the diffusion of soluble EDA (Swee et al., 2009). If this model is true, the prediction is that soluble agonists will never rescue zigzag hairs or any process that requires polarized expression of *Eda*.

Of note, a clinical trial to test the efficacy of a human form of Fc-EDA1 has recently been initiated in newborn patients with XLHED.

Pharmacological inhibitors of EDAR

Recombinant or endogenous EDA1 can be inhibited *in vitro* or *in vivo* with a soluble decoy EDAR-Fc fusion protein containing the extracellular, receptor-binding domain of EDAR (Fessing et al., 2006; Swee et al., 2009) (Fig. 1C). Function-blocking anti-EDA monoclonal antibodies have been raised in *Eda*-deficient mice. They block EDA at close to stoichiometric ratio and can induce ectodermal dysplasia in mice (Kowalczyk-Quintas et al., 2014). The availability of monoclonal mouse antibodies able to block and/or activate EDAR signalling *in vivo* will be useful to study this pathway during or after development.

Conclusion

Ectodysplasin A1 and its receptor EDAR are important for the development and function of several, but not all, ectodermal appendages. This system is part of a complex process of cell communication and differentiation involved in morphogenesis, which has been best characterized during development of mouse teeth and primary hairs, but whose common themes also apply at least in part to other structures and species. EDAR signalling occurs rather late in development and affects a defined set of organs. Available genetic data identified much of the mechanism and implications of EDAR signalling. The availability of pharmacological modulators of the pathway raises hope to use this knowledge for the treatment of certain forms of ectodermal dysplasia. Modulation of the EDAR signalling pathway may also find applications in pathologies involving *Edar*-regulated glands and organs.

Table 1: Phenotype associated with altered expression of genes of ectodermal appendages, by alphabetical order of the genes.

Protein *Gene modification*	Remarks, phenotype	Ref
β-catenin *one stabilized allele (Δex3) in K14 tissues*	Lethal at birth. Early hair placode initiation. Increased number of hair placodes (even without *Eda*), but suppressed follicle down growth. Limb and craniofacial abnormalities. Multiple mono-cuspid teeth.	(Jarvinen et al., 2006; Liu et al., 2008; Narhi et al., 2008; Zhang et al., 2008)
β-catenin *K14-Cre;β-catenin* (medium Cre)	Viable. Block of hair development in *β-catenin*-deleted skin areas. Body hair that form are lost at 2 week of age.	(Huelsken et al., 2001; Zhang et al., 2009)
β-catenin *K14-Cre;β-catenin* (early Cre)	Viability not mentioned. Block of hair development. Loss of patterned β-catenin activity in E13.5 to E14.5 dermis.	(Huelsken et al., 2001; Zhang et al., 2009)
ActivinβA *ActivinβA-null*	Lethal at P1. No whiskers. Incisor and mandibular molar failed to develop beyond the bud stage. Maxillary molars unaffected.	(Ferguson et al., 1998; Jhaveri et al., 1998; Matzuk et al., 1995)
ActivinRIIA+B *ActRIIB-null +ActRIIA-het*	Lethal at birth. Similar to *ActivinβA*-null. Partial penetrance of tooth phenotype.	(Ferguson et al., 2001)
BMPR1a *K14-Cre;Bmpr1a* (early Cre)	Lethal at P1. Severe limb defects. Opened eyes. Accelerated hair placode development, but normal follicle number at birth. Mammary glands present. Tooth development arrest at bud stage (E13.5). No teeth.	(Andl et al., 2004)
BMPR1a *K14-Cre;Bmpr1a* (medium Cre)	Lethal around P4. Ablation almost complete at E15.5. Normal limbs and eyes, runted, lack external hair, whiskers and teeth. Defective hair follicle (outer root sheath present, but inner root sheath does not form). *Lef1* is expressed, but not Wnt-target genes.	(Andl et al., 2004; Kobielak et al., 2003)
BMPR1a *K14-Cre;Bmpr1a* (late Cre)	Survive up to several months. Runted. Decreased hair.	(Andl et al., 2004)
BMPR1a *Brn4-Cre;Bmpr1a*	Survive 2 weeks. Deletion in neural tube, limb epithelium, ventral epidermis. Accelerated hair placode development, no hair shaft → no external hair in mid ventrum. CNS defects.	(Andl et al., 2004)
BMPR1a BMR1b *Brn4-Cre;Bmpr1a + Bmpr1b*	Same as Brn4-Cre32;BmprIa only (→ Bmpr1a and Bmpr1b are not redundant).	(Andl et al., 2004)
DKK1 *K14::Dkk1* *high expression*	Lethal at birth. Expressed from E9.5. No hair development. No vibrissae. No *Edar* or *Lef-1* expression. Block of molar and incisors development at the bud stage. Mammary gland development arrest at the bud stage.	(Andl et al., 2002; Liu et al., 2008; Zhang et al., 2009)
DKK1 *Foxn1::Dkk1* *high expression*	Viable. Gene expression in hair cortex, and weakly in epidermis. No hair follicles, except few guard hair follicles with no shaft (because *Foxn1* promoter is activated late).	(Hammerschmidt and Schlake, 2007)
DKK1 *Foxn1::Dkk1* *mild expression*	Viable. Gene expression from E14 to E14.5. Focal alopecia behind the ears. No sweat glands. Thin hair without bends, no zigzag hair. Guard hairs present but shorter.	(Hammerschmidt and Schlake, 2007)

EDA *Eda-deficiency*	Tabby mice. Viable. Alopecia behind the ears, no tail hair, no sweat glands, no mebomian glands, no tracheal glands, lack of several other glands, abnormal hair coat (only zigzag hair with abnormal awl-like morphology), smaller molars with fewer cusps, third molar sometimes lacking. Small eye opening, thickened eyelids.	(Gruneberg, 1971; Hammerschmidt and Schlake, 2007; Pispa et al., 1999)
EDA1 *Tet off::Eda1* *K14::Eda1*	Viable. No kinks in zigzag hairs, sebaceous gland hyperplasia, supernumerary teeth and nipples, continuous hair placode formation from E14 to birth.	(Cui et al., 2003; Mustonen et al., 2003)
EDA2 *Tet off::Eda2* *K14::Eda2*	Viable. No phenotype in ectodermal appendages.	(Cui et al., 2003; Mustonen et al., 2003)
EDAR *Edar-deficiency*	Downless and Sleek mice. Viable. Same phenotype as *Eda-*deficient mice.	(Headon and Overbeek, 1999)
EDAR *Tg951 (~20 copies of YAC transgene)*	Sebaceous and mebomian glands are enlarged, salivary and mammary glands are more elaborately and more branched, enlarged hair follicles, thicker hair fiber.	(Chang et al., 2009; Mou et al., 2008)
EDARADD *Edaradd-null*	Crinkled mice. Deletion. Viable. Same phenotype as *Eda-*deficient mice.	(Falconer et al., 1951; Headon et al., 2001)
FGF20 *Fgf20-null* *(knock-in with βGal)*	Smaller molars, reduced crown area. No guard hair, reduced numbers of awl and auchene hair, normal zigzag hair. Placodes are generated for missing hair, but no dermal condensation form underneath.	(Haara et al., 2012; Huh et al., 2013)
FGF20 *Fgf20-deficiency*	Scaleless mutant in chicken. Non-sense mutation in *Fgf20*. No feathers and scales.	(Wells et al., 2012)
Follistatin *K14::follistatin*	Viable. Inhibition of ameloblasts differentiation in incisors. No enamel.	(Wang et al., 2004)
Follistatin *Follistatin-null*	Lethal soon after birth. Retarded growth. Diaphragm, muscle and skeletal defects. Shiny taut skin. Thin and curly whiskers. Molar cusps defects, ameloblasts differentiate ectopically on the lingual enamel-free surface of the incisor.	(Jhaveri et al., 1998; Matzuk et al., 1995; Wang et al., 2004)
GLI2, GLI3 *Gli2-null, Gli3-null*	Lethal around E10.5, few embryos survive to E14.5. Incisors blocked at bud stage, no sign of molar development.	(Hardcastle et al., 1998)
IκBα *ki of IκBαΔN* (super-repressor) *in β-catenin locus*	Viable. Alopecia behind the ears, no tail hair, no sweat glands, no mebomian glands, abnormal hair coat (single awl-like hair type). Reduced number of hair follicles. Delayed outgrowth of incisors and molars. Abnormal incisor positioning. No lymph nodes, osteopetrosis, immuno-deficiency.	(Ohazama et al., 2004; Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 2006)
LEF-1 *K14::Lef1*	Viable. Aberrant patterning of the hair on the body. Inappropriate eruption of hair and tooth-like structures in the oral epithelium	(Zhou et al., 1995)
LEF-1 *Lef1-null*	Lethal at birth. Lack of vibrissae and body hair. Tail hair and sweat gland present. Tooth development arrest at bud stage. Lack of mammary gland	(van Genderen et al., 1994)
NEMO/IKKγ *mutations in human*	On the X chromosome. Complete deficiency is lethal. Heterozygous females develop incontinentia pigmenti. Males with hypomorphic mutations have hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID). Males with stop	(Smahi et al., 2002; Zonana et al., 2000)

	codon mutations have in addition osteopetrosis and lymphedema (OL-HED-ID).	
Noggin *K14::Noggin*	Viable and fertile. Chicken *Noggin*. All hair types are present. Too many awls and auchenes. Hypertrophic sebaceous glands. Shorter telogen phase. Compound follicles with 2-3 vibrissae. Ectopic cilia instead of mebomian glands, small eyelid opening, smaller footpads with hair instead of sweat glands. No claws. Increased size of external genitalia in both sexes.	(Plikus et al., 2004)
Noggin *K5::Noggin*	Increased size of anagen hair follicles, replacement of zigzag and auchene hairs by awl-like hairs (no more hair with bends).	(Sharov et al., 2006)
Noggin *Noggin-null*	Reduced hair placode number. Hair development arrest at bud stage.	(Botchkarev et al., 1999)
SHH *Foxn1::Shh*	Viable. Expression mainly in hair cortex. Attenuation of kinks in zigzag hair attributed to altered polarity of follicles.	(Hammerschmidt and Schlake, 2007)
SHH *Shh-null, and grafted embryonic null skin*	Lethal at birth. The first branchial arch giving rise to jaw mandibles does not form. Hair development blocked at placodes and dermal condensate stage. Reduced number of hair follicles.	(Chiang et al., 1999; St-Jacques et al., 1998)
SHH *K14-Cre;Shh*	Lethal at birth. Deletion at E12.5, (still expressed at E11.5). Opened eyes. No whiskers. Jaws present. Small and abnormally shaped incisors and molars. Defect at cap stage (E14.5), functional enamel knot, severe defect of epithelial cells downgrowth on the lingual side where Shh is normally expressed). Ameloblasts and odontoblasts still differentiate.	(Dassule et al., 2000)
SMAD2 *Smad2-het*	Lethal at birth. Similar to *ActivinβA*-null. Partial penetrance of tooth phenotype.	(Ferguson et al., 2001)
Sostdc1 *Sostdc1-null*	Viable. Enlarge enamel knot, supplementary incisors and molars, fused molars and cusps defect. Enlarge hair and mammary placodes. Ectopic whiskers.	(Ahn et al., 2010; Kassai et al., 2005; Narhi et al., 2012)
Sostdc1/Ectodin *K14::Sostdc1*	Viable. Reduced tooth size, loss of the M3 molar, and cusps defect. Abnormal development of hair follicle.	(Ahn et al., 2010; Ahn et al., 2013)
TRAF6 *Traf6-null*	Viable. No guard hair, sweat glands, sebaceous glands, mebomian glands, anal glands, and preputial glands. Alopecia behind ears, hairless tail with kink. Severe osteopetrosis, alymphoplasia, abnormal B cells, impaired IL1R and TLR signaling.	(Naito et al., 2002)

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FIGURE LEGENDS

Fig. 1: **Protein organisation and mode of action of the ligand and receptor pair EDA1-EDAR and some of their agonists and antagonists**.
A.- Structural organisation of EDA1, EDAR and EDARADD. HED- and STA-causing mutations are those listed in UniProt. DD: death domain.
B.- Principle of EDAR activation by endogenous EDA1.
C.- Principle of action of soluble agonists and antagonists of the EDA - EDAR pathway.
D.- Principle of *in utero* delivery of agonists and antagonists of EDAR signalling via transplacental antibody transport.

Fig. 2: **Naïve view of signalling pathways involved in hair and tooth morphogenesis at placode or bud stages**.
EDA and EDAR are important targets of canonical Wnt signalling in the epithelium. EDAR in turn stimulates NF-κB-mediated transcription of genes acting in major hair and tooth development pathways: Wnt (*Wnt10a/b* and the antagonist *Dkk4*), Hedgehog (*Shh*), TGFβ (the antagonists *Follistatin* or *CTGF*) and FGF (*Fgf20*). Components of these pathways and some of their relationships are also illustrated.

Fig. 3: **Long-term improvement of ectodermal dysplasia features by EDAR agonists in *Eda*-deficient mice**.
Long-term reversion of hair (tail, guard, retro-auricular), eye and functional sweat glands in a 21 months-old *Eda*-deficient mouse treated at days 8.5 and 13.5 of gestation with 4 mg/kg of an agonist anti-EDAR monoclonal antibody (mAbEDAR3 (Kowalczyk et al., 2011)).
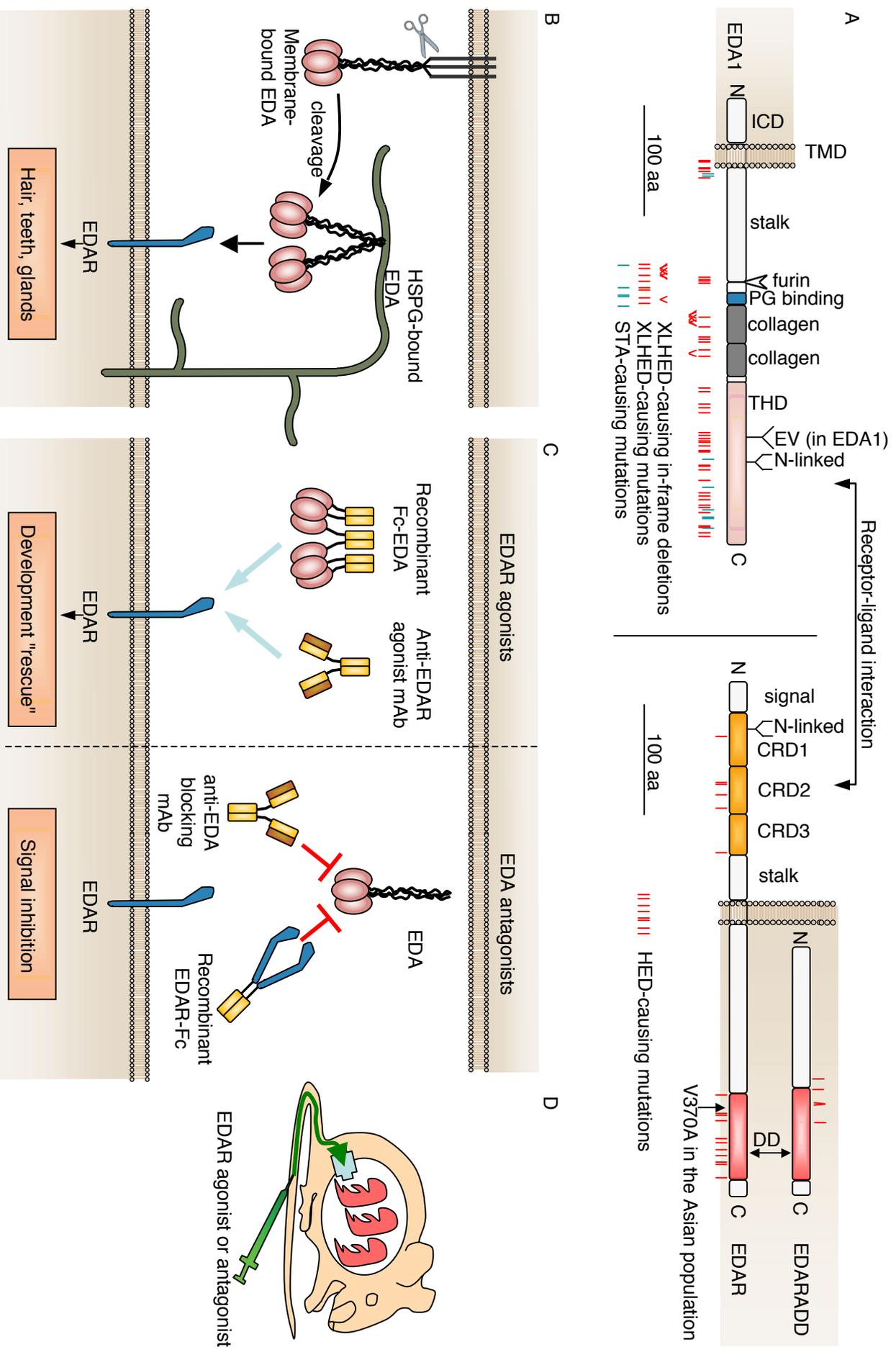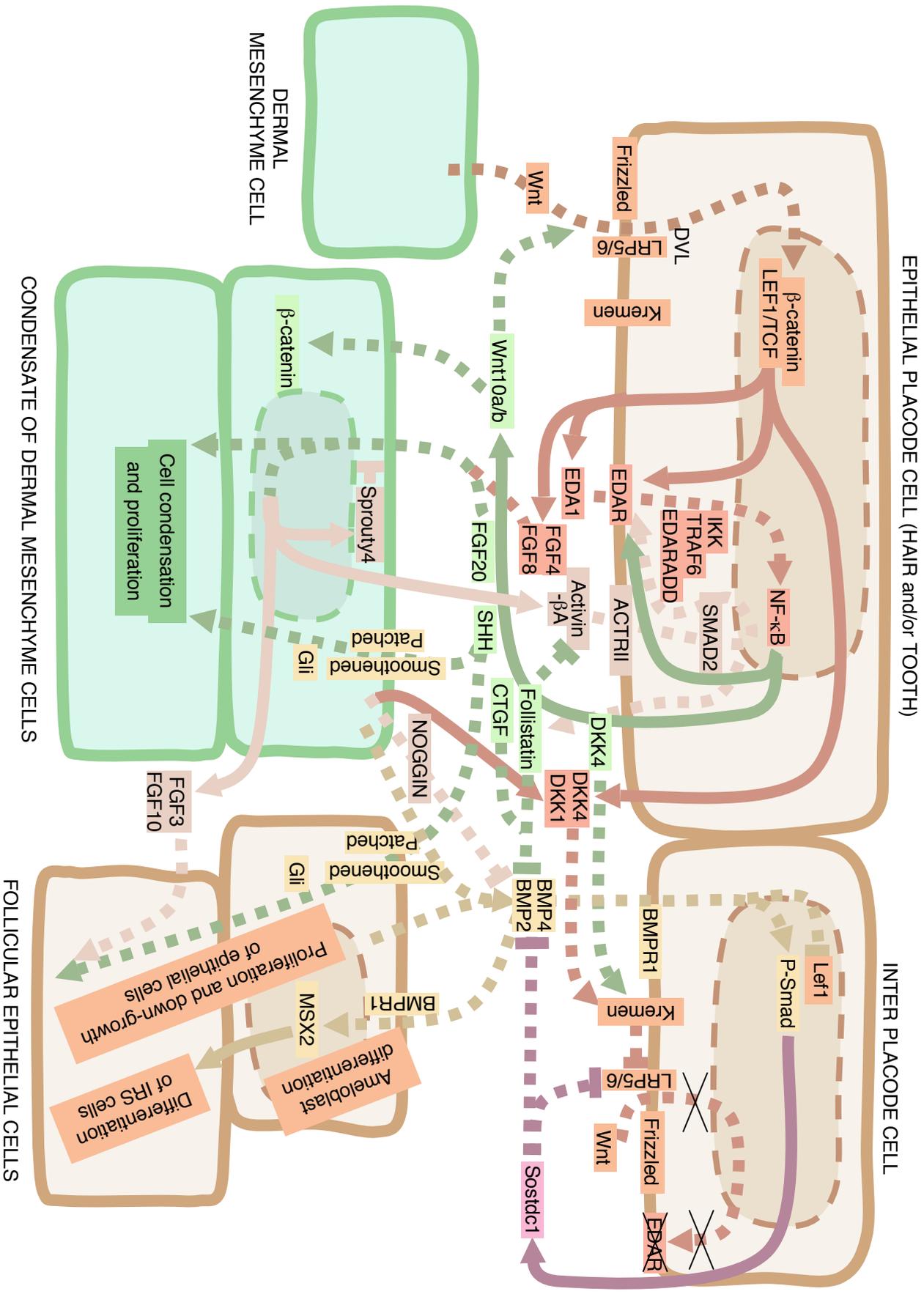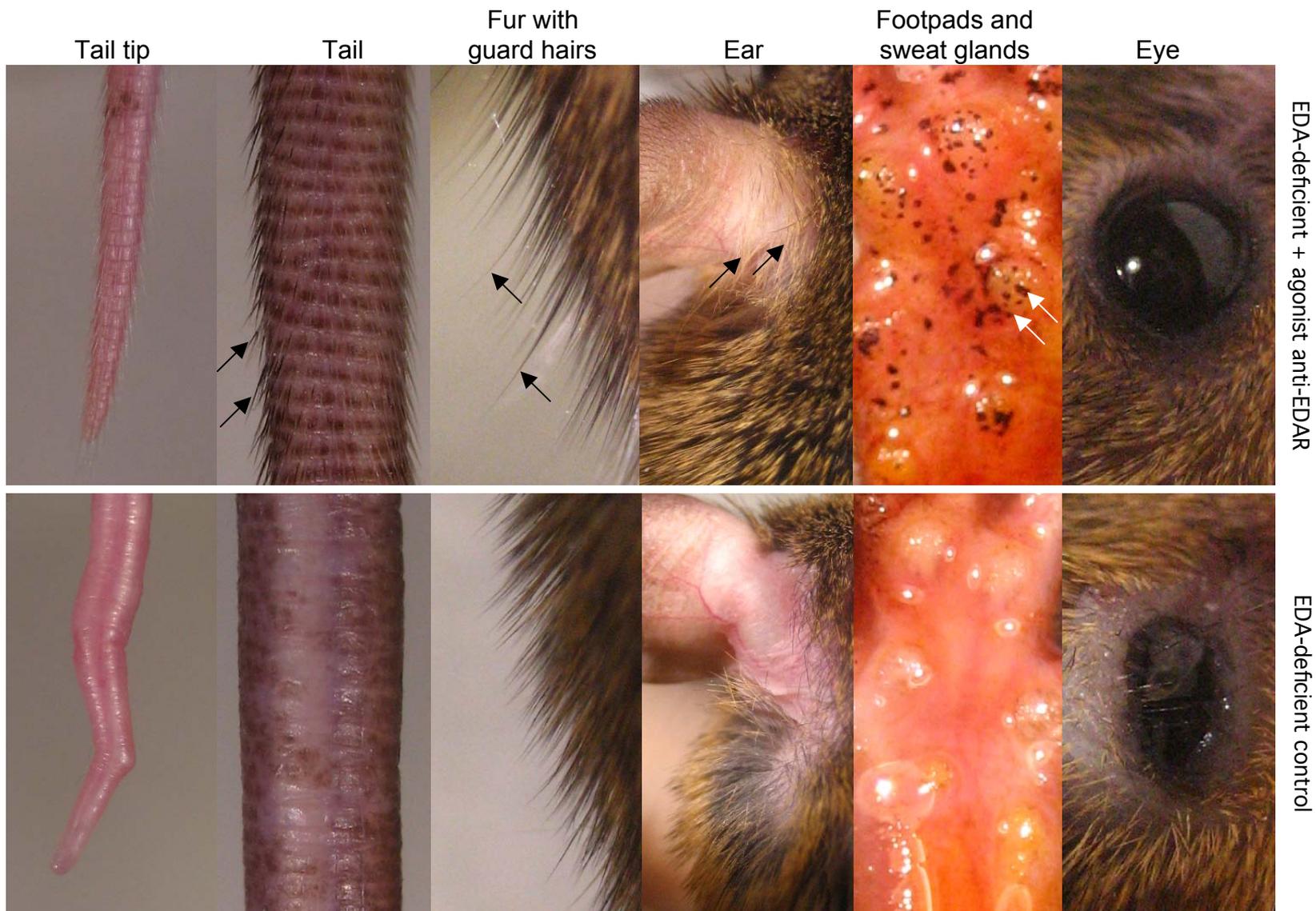
Figure 1

Figure 2

| Tail tip | Tail | Fur with guard hairs | Ear | Footpads and sweat glands | Eye |

EDA-deficient + agonist anti-EDAR

EDA-deficient control

Figure 3