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<td><strong>Science Writers</strong></td>
<td>Eva Marie Y. Moresco</td>
</tr>
<tr>
<td><strong>Authors</strong></td>
<td>Karine Crozat, Sophie Rutschmann, Bruce Beutler</td>
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<tr>
<td><strong>Illustrators</strong></td>
<td>Diantha La Vine</td>
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Cite this information as follows: Karine Crozat, Sophie Rutschmann, Eva Marie Y. Moresco, Beutler B. Record for *achtung2*, updated Apr 24, 2018. MUTAGENETIX (TM), B. Beutler and colleagues, Center for the Genetics of Host Defense, UT Southwestern Medical Center, Dallas, TX. URL: mutagenetix.utsouthwestern.edu

1 of 13

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Phenotypic Description

The *achtung2* phenotype was identified among G3 ENU-mutagenized mice. *Achtung2* mice are characterized by a lack of fur behind the ears, an unusually stiff posturing of the tail, a crook at the tip of the tail, and abnormally short teeth. The animals resemble *achtung* or *tabby/Sleek/downless* mice, which have mutations in components of the EDAR signaling pathway.

Nature of Mutation

The *achtung2* mutation corresponds to a C to A transversion at position 1305 of the *Edar* transcript on Chromosome 10, in exon 12 of 12 total exons.

1289 AGCCCCACCGAGTTGCGCGTTTACTGCCTTGAG
343  -S--P--T--E--L--P--F--D--C--L--E--

The mutated nucleotide is indicated in red lettering.

Protein Prediction

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The EDAR protein consists of 448 amino acids, and belongs to the tumor necrosis factor receptor (TNFR) superfamily (1). EDAR is highly conserved between vertebrate species, including fish, frogs, chicken, dogs, cattle and chimpanzee (2); the mouse and human proteins are 91% identical (3). Two additional EDAR family members exist: XEDAR (X-linked ectodysplasin-A2 receptor) (4) and Troy (5). Mouse EDAR is a type I transmembrane protein (extracellular N terminus), containing a cleavable signal peptide at the N terminus, followed by a 159 amino acid TNFR-like extracellular domain (Figure 1). The human protein does not appear to contain a signal peptide (3). The extracellular domain, responsible for ligand binding, contains 14 cysteine residues residing in three cysteine-rich domains (CRDs) similar to those of TNFR; the 6 cysteines closest to the N terminus follow the TNFR consensus sequence most closely (1;3). Ligand binding experiments using recombinant chimeric receptors containing combinations of the three CRDs from EDAR or XEDAR, and homology modeling of EDAR based on the structure of TNFR1, suggest that the specificity of EDAR-ligand interactions is determined by CRD3, the most membrane-proximal CRD (6).

Following a single transmembrane domain, the 237 amino acid intracellular region of EDAR contains a segment near the C terminus with homology to the death domain of TNFR and other TNFR-like proteins (1;3). The death domain was first identified in proteins involved in cell death induction, but it is now known to function as a protein-protein interaction domain that mediates homotypic interactions with other death domain-containing proteins in order to propagate signaling. In the case of EDAR, it does not seem to connect to the apoptotic machinery.

The \textit{achtung2} mutation results in the substitution of proline 349 by glutamine. This residue is located just N terminal to the death domain.

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Expression/Localization

RT-PCR analysis detects human EDAR transcript in embryonic and fetal skin at 11-15 weeks estimated gestational age, and in a 42-53 day old craniofacial cDNA library (3). Consistent with these data, mouse Edar transcript is detected in basal cells of the epidermis, where it is uniformly distributed before follicle initiation (~embryonic day 13) and subsequently elevated and restricted in follicle epithelial cells (~embryonic day 15-17) (1;4). EDAR is also detected in lacrimal, mammary, and salivary glands (7). Surprisingly, when expressed in 293A cells, EDAR protein is primarily expressed intracellularly, a localization that has been reported for other TNF receptors (8). It was postulated that EDAR may be delivered to the cell surface in a regulated manner (8).

In humans, Northern blot analysis also detects EDAR transcript in fetal kidney and lung (3).

Background

The four mutant mouse phenotypes Tabby (Tb), downless (dl), Sleek (Sl) and crinkled (cr), first reported in the 1950s, are characterized by lack of hair on the tail and behind the ears, lack of or abnormal morphology of incisors, a reduced number of vibrissae, and occasionally a kinked tail tip [(9-11) and reviewed in (7)]. Further study demonstrated that sweat glands (normally on the footpads), lacrimal, Meibomian and submandibular glands, and three of four types of mouse hair, are lacking in these mutants (7). Human patients also develop a similar syndrome, hypohidrotic (or anhidrotic) ectodermal dysplasia (HED), which may be transmitted in an autosomal dominant (OMIM #129490), autosomal recessive (OMIM #224900), or X-linked recessive (OMIM #305100) manner. Humans with HED typically have missing or sparse hair, missing or misshapen teeth, and absent or reduced ability to sweat. The reduced ability to sweat can cause hyperthermia, and results in a 30% mortality rate in children up to age 2 if the condition is not recognized (12). HED patients also have dry skin, eyes, airways and mucous membranes. Recently, ocular surface abnormalities (corneal lesions, inflammation) likely due to a lack of Meibomian glands were reported in both human HED patients and in Tabby mice (13;14).

Mutations in EDA (15), EDAR (3) and EDARADD (16) are now known to cause HED in humans, as well as the Tb (17;18), dl or Sl (1), and cr (16;19) phenotypes, respectively, when the orthologous genes are mutated in mice. In humans, mutations of the X-linked EDA gene cause the majority of cases of HED. Male patients display a similar severity of phenotype, but heterozygous females with EDA mutations vary considerably in their symptoms due to varying levels of X-inactivation of the chromosome containing the mutated gene (20;21). Mutations in EDAR account for approximately
25% of cases, and can cause both autosomal dominant or recessive forms of HED (22;23). As in
humans, Eda is an X-linked gene in mice, and female Agouti (A) mice (normally with light/yellow
hair) heterozygous for Tb, in contrast to hemizygous males, grow “tabby” colored fur (containing
striping of variable degree) in a mosaic pattern, giving an appearance of transverse stripes of light-
colored normal and dark hair (9;36). Loss of yellow pigment results in black hair in areas of skin
containing the mutation in these mice (36). The underlying mechanism by which Eda affects
pigmentation in A mice is unknown, and interestingly, no pigmentation defects have been reported in
humans with EDA mutations. EDA (ectodysplasin), EDAR and EDARADD (EDAR-associated death
domain) form the ligand, receptor and adaptor proteins, respectively, of a TNF-related signaling
pathway specific to the development of so-called skin appendages, ectoderm-derived tissues
including hair follicles, nails, teeth and exocrine glands in mammals.

The ligand for EDAR, EDA, is a trimeric type II transmembrane protein (extracellular C terminus)
with extracellular collagen and TNF domains (24). Cleavage by furin near the collagen segment
releases soluble EDA to bind to EDAR (25). The gene encoding EDA is alternatively spliced to form
several different transcripts [at least six in humans (26) and nine in mice (27)]. The two longest
isoforms, EDA-A1 (391 aa) and EDA-A2 (389 aa), account for approximately 80% of the total EDA
transcripts in mouse keratinocytes (27), and appear to be the functional forms (26). EDA-A1 and
EDA-A2 differ by only two amino acids in the receptor-binding region of the protein: E308 and
V309 are lacking in EDA-A2 (4). Strikingly, this two amino acid difference accounts for an absolute
specificity of EDA-A1 and EDA-A2 for different receptors, EDAR and XEDAR, respectively (4). As
mentioned above (Protein Prediction), the third CRD of EDAR is thought to recognize distinct
structural features conferred by the presence of E308 and V309 in EDA-A1, as is the third CRD of
XEDAR for structural features of EDA-A2 (6).

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6 of 13
(Generated on Sep 11, 2018)
Figure 2. EDA Pathway. Ectodysplasin-A (EDA) is a trimeric type II membrane protein of the tumor necrosis factor family that signals during the development of ectodermal organs. EDA is cleaved by furin, releasing soluble EDA-1 that binds to EDAR. The EDARADD adapter is recruited to EDAR via death domain (DD) interactions. TRAF6, TAK1, and TAB2 are also recruited to the complex. IKK activation occurs upon polyubiquitination of NEMO by TRAF6 or phosphorylation of IKK by activated NIK. Once, activated IKK phosphorylates IκB, leading to ubiquitination and degradation of IκB, and release of NF-κB for translocation to the nucleus where it activates target genes. CYLD may inhibit this pathway by deubiquitinating TRAF6. This image is interactive. Click on the image to view mutations found within the pathway (red) and
Like signaling from TNFR and the Toll-like receptors, signaling from EDAR is mediated by the TRAF6/TAK1/TAB2 complex, and ultimately activates NF-κB to stimulate gene transcription. EDA binds to EDAR as a trimer, leading to the recruitment of its specific adapter, EDARADD, through receptor/adapter death domain interactions (16). EDARADD can interact with TRAFs 1, 2 and 3 in GST pulldown experiments (16), but only TRAF6 has been detected in developing hair follicles (expression of other TRAFs in ectodermal tissues has not been tested) (28). EDARADD has recently been shown to coimmunoprecipitate from 293 cells with TRAF6, TAB2 and TAK1 (29). In addition, TRAF6-deficient mice develop a similar, but not identical phenotype, to that of Tabby or downless mice, with lack of hair on the tail and behind the ears, and defects in teeth, hair follicles, and exocrine glands (28). These data support the conclusion that EDARADD recruits a complex containing TRAF6, TAB2, and TAK1.

Transfection of EDAR into 293E cells results in dose-dependent activation of NF-κB, and this response is drastically reduced when cells are transfected with mutant forms of EDAR containing the dl mutation or lacking the death domain (4;30). In vivo, loss-of-function mutations in the human or mouse IKK complex subunit IKK?/NEMO result in HED (along with immunodeficiency) due to impaired NF-κB signaling (see panr2) (31;32). Mice expressing a constitutively active form of I?B? develop a phenotype similar to Tabby and downless (33). Together, the data suggest that the TRAF6/TAK1/TAB2 complex activates the IKK complex to phosphorylate I?B?, which then releases NF-κB for translocation to the nucleus and activation of gene expression (16;29;31;34).

Interestingly, although XEDAR signaling can also activate NF-κB in vitro (4), it appears that XEDAR has no role in skin appendage development, as XEDAR-deficient mice have normal ectodermal-derived organs (35). Troy, the third EDAR family member, is also expressed in skin, including hair follicles, and activates NF-κB when overexpressed in 293T cells (5). However, an ENU-induced mouse mutant of Troy that is unable to activate NF-κB has normal hair follicles, hair shafts, hair type composition and sweat glands (2).

The downstream targets of EDAR signaling are reported to include proteins of the Wnt, Sonic hedgehog (Shh), bone morphogenetic protein (BMP) and lymphotoxin- ? (LT?) pathways, which were identified by comparative gene expression analysis of Tabby and wild type skin [reviewed in (2)]. The specific role of these pathways in skin appendage development and the mechanisms by which they interface with EDAR signaling not well understood.
The *achtung*2 mutation results in the substitution of proline 349 by glutamine. This residue is located just outside the death domain (aa ~356-431) (1). Since proline residues typically facilitate kinks in polypeptide secondary structure, its substitution in *achtung*2 mice may disrupt such folding in this region of the protein. Aberrant folding of the death domain may prevent recruitment and subsequent signaling by EDARADD. Several EDAR mutations causing HED in humans or mice disrupt the function of the death domain. For example, the *dl* mutation is an E to K point mutation of residue 379, within the death domain, and the *Sl* mutation truncates EDAR just before the death domain (1). It appears that most HED-causing mutations in human EDAR occur within either the death domain or the ligand binding domain (3;22).

**Genotyping**

*Achtung*2 genotyping is performed by amplifying the region containing the mutation using PCR, followed by sequencing of the amplified region to detect the single nucleotide change. This protocol has not been tested.

**Primers for PCR amplification**

Ach2(F): 5’- TCCCAACCTCAGCTTTTAGCAGTG -3’  
Ach2(R): 5’- GTCCAAGACAACCTCTTCAGGACGC -3’

**PCR program**

1) 94°C 2:00  
2) 94°C 0:30  
3) 56°C 0:30  
4) 72°C 1:00  
5) repeat steps (2-4) 29X  
6) 72°C 7:00  
7) 4°C ?

**Primers for sequencing**

Ach2_seq(F): 5’- GTGTACTAGCTCTGGACTTAACATC -3’  
Ach2_seq(R): 5’- TGCACCAACTTTTGAGCAG -3’

The following sequence of 684 nucleotides (from Genbank genomic region NC_000076 for linear...
DNA sequence of *Edar* is amplified:

72167 tccc aacctcagct
72181 tttagcagt tactagctct ggacatcac tcacattgct ggtataacttt gaataccgaa
72241 ccaatacctag ataaatgtc agaagactatt tagaactctc
72301 attaatgacttactgatgt actattgag ctgctgctgag ctgctgactt
72361 cgctctctct ctgctgctgag ctgctgctgag ctgctgctgag
72421 tgcgtgtgacttctgctgag ctgctgctgag ctgctgctgag
72481 ctgggttctct ctgctgctgag ctgctgctgag ctgctgctgag
72541 cagctgctgag ctgctgctgag ctgctgctgag ctgctgctgag
72601 cagctgctgag ctgctgctgag ctgctgctgag ctgctgctgag
72661 cagctgctgag ctgctgctgag ctgctgctgag ctgctgctgag
72721 cagctgctgag ctgctgctgag ctgctgctgag ctgctgctgag
72781 cagctgctgag ctgctgctgag ctgctgctgag ctgctgctgag
72841 ctgctgctgag ctgctgctgag ctgctgctgag ctgctgctgag

PCR primer binding sites are underlined; sequencing primer binding sites are highlighted in gray; the mutated C is shown in red text.

**References**

20. Bartstra, H. L., Hulsmans, R. F., Steijlen, P. M., Ruige, M., de Die-Smulders, C. E., and


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