Functional Analysis of Ectodysplasin-A Mutations Causing Selective Tooth Agenesis

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ABSTRACT

Mutations of the Ectodysplasin A (*EDA*) gene are usually associated with the syndrome Hypohidrotic Ectodermal Dysplasia (HED, MIM 305100) but they can also manifest as selective, non-syndromic tooth agenesis (MIM300606). *In vitro* functional analysis of one novel and five known selective tooth agenesis causing *EDA* mutations, which are all located in the C-terminal tumor necrosis factor (TNF) homology domain of the protein, reveals that expression, receptor binding or signaling capability of the mutant EDA1 proteins is only impaired while syndrome-causing mutations have been previously shown to abolish EDA1 expression, receptor binding or signaling. Our results support a model in which the development of the human dentition, especially of anterior teeth requires the highest level of EDA-receptor signaling while other ectodermal appendages, including posterior teeth have less stringent requirements and form normally in response to EDA mutations with reduced activity.

INTRODUCTION

Tooth development is largely under genetic control and all familial cases as well as the majority of sporadic cases of congenital tooth agenesis are likely to be caused by sequence variations in one of the many genes that are involved in tooth development (1). Classically, a distinction has been made between non-syndromic and syndromic tooth agenesis. Hypohidrotic Ectodermal Dysplasia (HED) is such a syndrome which features hypoplasia of most epithelial appendages including variable numbers of missing teeth, sparse hair, hypoplastic or aplastic sweat glands leading to hypohidrosis with potentially life-threatening hyperthermia. Non-syndromic tooth agenesis on the other hand, has been defined as affecting predominantly the dentition and involving primarily tooth-specific genes (2-4).

The distinction between syndromic and non-syndromic tooth agenesis is becoming less apparent as genetic knowledge advances. Mutations in the Muscle Segment Homeobox 1 gene (MSX1) for example cause not only selective tooth agenesis, but are also involved in orofacial cleft syndromes (5). Not only do the more "tooth-specific" genes contribute to non-dental problems, but the reverse has also been observed: There have been several previous reports about isolated tooth agenesis in families with mutations in the Ectodysplasin-A (*EDA*) gene (6-12) which is usually responsible for the HED syndrome (13).

The *EDA* gene occupies a 425 kb segment of the long arm of the X-chromosome (Xq12-13.1) (14) and encodes a protein which belongs to the tumor necrosis factor (TNF) superfamily of ligands. Eight isoforms of the *EDA* transcript can be created by differential splicing of the 12 exons. The longest splice form encodes a 391 amino acid protein with a transmembrane domain, a furin cleavage site, a collagen type repeat area in the middle and a C-terminal TNF-like structure (15-17). Upon proteolytic processing at the furin consensus site, the C-terminal portion of the protein containing the collagen domain and the TNF homology domain is released as a soluble, trimeric ligand (18). Only two splice isoforms of EDA contain the receptor-binding TNF homology domain: EDA1 which binds the EDA-receptor EDAR, and EDA2, a two amino acid shorter variant that binds exclusively to a receptor called XEDAR (19). The TNF homology domains of both EDA1 and EDA2 have been crystallized as homotrimers, but further multimerization through interactions of the collagen domain appear to be functionally important.

Both receptors, EDAR and XEDAR activate the NF-_B pathway but only the EDA1/EDAR interaction seems relevant for the development of ectodermal appendages. Indeed, mutations in EDA1 as well as EDAR can cause a HED syndrome that is phenotypically indistinguishable except for the different inheritance pattern, X-linked versus autosomal respectively (20). In addition, XEDAR mutations have not yet been identified in HED or any other condition, and XEDAR-deficient mice are normal and display none of the phenotypes characteristic of ectodermal dysplasia (21).

Dozens of different *EDA* gene mutations have been identified in patients with the HED syndrome. These mutations range from exon deletions and frameshifts to conservative replacements of single amino acids and may be located in any of the three main functional domains of the EDA protein, furin cleavage site, the collagen-like multimerization domain or the TNF homology domain. Phenotypically, the HED

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syndrome is diverse with a broad range of intra- and interfamilial variation in severity. However, despite the great number of different EDA mutations that have been studied so far, no genotype/phenotype correlations have been uncovered.

Previous investigations of the impact of *EDA* missense mutations on different aspects of protein function have shown that proteolytic processing, glycosylation, multimerization and solubility or solely receptor binding can be affected by the different mutations (8, 18, 22). Ultimately, most of the mutations could be predicted to lead to an elimination of receptor signaling; only one mutation was shown to possess residual receptor–binding activity. Interestingly, this mutation was found in a HED family whose main complaint was tooth agenesis (8). Here we describe an additional family with X-linked recessive, non-syndromic tooth agenesis that can be linked to an EDA mutation and present a functional analysis of this EDA mutation in comparison with other tooth agenesis and HED causing mutations in the TNF homology domain of EDA.

RESULTS

Mutation Detection in a Tooth Agenesis Family
The pedigree of this Caucasian tooth agenesis family shows a typical X-linked recessive pattern of inheritance, affecting only males (Fig. 1A). Fig. 1B gives an overview of the dental phenotype of the affected participants. In all cases two to six primary or permanent incisors are missing, with the exception of maxillary central incisors. First or second premolars and second molars of the permanent dentition may be missing as well. Carrier female family members report full dentition without shape or size abnormality.

Since the pedigree pattern was strongly suggestive of an X-linked condition and the *EDA* gene on Xq12-13.1 is associated with a syndrome that includes tooth agenesis, a candidate gene approach was pursued and led to the detection of a T_C transition at nucleotide 1336 of the deduced cDNA sequence. The nucleotide replacement produces a conservative change of the surface-expressed Valine 365 to Alanine (Fig. 2A and B). The T_C nucleotide change also introduces a HhaI restriction site into the last exon of the *EDA* gene, which allowed the testing of all twenty-six available family members for the V365A mutation by restriction fragment length polymorphism. A perfect segregation of the T to C transition with the phenotype became apparent: Affected males have a single mutant copy, carrier females have one mutant and one wild type copy and unaffected probands have only wild type copy(s) (Fig. 1A).

A common polymorphism at this site of the *EDA* gene was excluded by testing seventy-nine controls with a total of 144 X-chromosomes (65 females, 14 males) for the T to C transition at nucleotide 1336. None of these unrelated controls showed the mutation. We conclude that the V365A substitution is the likely cause for the tooth agenesis in this family.

In order to evaluate the phenotype further, participating family members were asked to complete a detailed two-page questionnaire (summarized in Table 1). The only symptoms reminiscent of HED appear to be a tendency for fine, curly hair, early balding (in their middle to late twenties) and thin skin. Heat intolerance is reported by some of the affected males but also by unaffected family members. The condition has been observed for four generations in this family but was never diagnosed as ectodermal dysplasia because of the uncharacteristically mild systemic features which are not significantly different from random variability in the general population. We conclude that this family's features fit the designation X-linked Hypodontia MIM300606.

Functional Characterization of Mutant Protein Constructs
The V365A mutation and the five other mutations (8-12) which cause selective tooth agenesis are located in the COOH- terminal quarter of the TNF-like receptor binding domain (Fig. 2A), and cluster close to the interface of two adjacent monomers where receptor binding is predicted to occur (30) (Fig. 2B).

All six mutations were tested for expression, glycosylation, cellular export, quantitative receptor binding and signaling ability as previously described for the S374R mutation (8). Fig. 3A shows

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normalized amounts of wild type and mutant EDA1 and EDA2 with the tooth agenesis causing mutations V365A, Q358E, S374R, D316G, T338M, M364T, or the HED-causing mutations Y343C and H252L. The two bands in each lane represent the glycosylated and the unglycosylated polypeptides of each construct, as demonstrated by peptide-N glycanase F digestion (Fig 3B). Although all nine protein constructs are expressed, glycosylated and exported out of the cells, there are a few marked differences. In mutant Q358E, the glycosylated band is consistently over-represented compared to the lower, non-glycosylated band. This may indicate a solubility problem of the unglycosylated form, causing it to be retained in the cell. Indeed, less than 10% of the tooth agenesis-causing mutant Q358E is recovered from cell supernatants. Secretion problems are not detectable in the other mutants, with the exception of the HED-causing Y343C variant which is also mostly retained in the transfected cells, consistent with our previous report (8) (Fig. 2D).

The relative amounts of Flag-tagged wt and mutant proteins were carefully determined in order to exclude that small differences in receptor binding activity are due to a difference in ligand concentration (Fig. 3C). Indeed, the quantitative ELISA (Fig. 4A, upper panel) shows that the binding of all hypodontia-causing EDA1 proteins to the receptor EDAR is variably reduced compared to wt, but not abolished. In contrast, EDA1 carrying either of the two HED-causing mutations Y343C and H252L loose all receptor-binding capability, consistent with the results that we previously obtained by testing HED-causing mutants (8). The affinity of the corresponding EDA2 proteins to XEDAR (Fig. 3E) is only slightly reduced, if reduced at all, for 5 of the 6 tooth agenesis-causing mutants (Fig. 4A, lower panel).

We next investigated the ability of EDA1 wt and mutant constructs to trigger a surrogate, multimerization-dependent signaling pathway in Jurkat T cells engineered to express an EDAR:Fas fusion receptor. In this system a functional EDA1 ligand can induce cell death via the Fas pathway; however this requires prior cross-linking of the EDA1 ligand with anti-Flag antibodies in analogy to the cross-linking of FasL, which is required for signaling in the original FasL/Fas pathway (23). Figure 3F shows that cross-linked wt EDA1 can induce cell death in this assay. The Q358E mutant is the only one showing appreciable, yet reduced activity in this assay. Surprisingly, V365A and M364T are mostly inefficient in the cell death-induction assay indicating that the signaling capacity of the mutated proteins is impaired even though cellular production, export and receptor-binding are readily detectable. S374R and T338M have hardly any activity in the EDAR-Fas signaling assay, in line with their severely reduced receptor binding capacity. Our assays would predict the D316G mutation to have the most severe effect and according to the published phenotype data this appears to be the case (9).

DISCUSSION

The development of teeth depends on a highly coordinated interaction of multiple signaling events between ectodermal and underlying mesenchymal cells, similar to the development of other skin appendages like hair follicles, nails and glands. A multitude of genes are known to be expressed in the developing tooth, but so far only four of them have been associated with selective, non-syndromic tooth agenesis in humans: 1) The Paired Box 9 gene (*PAX9*) is strongly expressed in developing teeth. Heterozygous mutations in *PAX9* cause predominantly molar tooth agenesis. 2) MSX1, a homeobox protein, can cause a slightly different phenotype with more premolar involvement. It may also contribute to Witkop (24) and cleft lip/palate syndromes. 3) Axis Inhibitor 2 (AXIN2) is a signaling molecule in the "wingless-type MMTV integration site family" (WNT) pathway which starts operating very early in tooth placode formation. Autosomal dominant mutations in exon 7 of *AXIN2* have been shown to cause not only a severe mixed pattern of tooth agenesis but also intestinal polyposis and predisposition to colon cancer. 4) The *EDA* gene, known to be affected in most patients with the HED syndrome, is also implicated in selective tooth agenesis. The hypodontia phenotype in EDA-associated isolated tooth agenesis seems to favor the lack of incisors but other teeth are involved as well.

However, these four genes are responsible for tooth agenesis in only a fraction of all observed hypodontia cases. Many more genes remain to be discovered; and so far only a few functional analyses of tooth agenesis causing mutations are available to explain the pathogenesis of congenital hypodontia.

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Most of the known EDA mutations affect a variety of ectodermal appendages like hair, teeth and glands. Absent or severely decreased sweating can lead to hyperthermia and death during minor febrile infections in childhood. Other problems caused by glandular hypoplasia are constipation, respiratory infections, insufficient production of saliva and lacrimal fluid, earwax impaction, frequent middle-ear infections, under-developed mammillae and lactation difficulties in affected females. Hair and eyebrows are sparse or missing. Some affected males lack all teeth, some only a few, but on average twenty-two permanent teeth are missing, not counting third molars (25). Shape abnormalities include smaller size, peg shape, reduced cusp number and root abnormalities. The facial features may be slightly dysmorphic with frontal bossing and depressed nasal bridge.

HED often manifests with variability among affected males of the same family. Carrier females generally show a much milder phenotype with only one or two teeth missing, slightly smaller teeth, sparse hair or no symptoms at all. Mutations in all portions of the gene can cause ectodermal dysplasia, with no apparent correlation between location of the mutation and severity of the phenotype.

The V365A mutation reported here is located in the C-terminal TNF-like portion of the protein, as are most of the other tooth agenesis-causing mutations including three newly described ones (A259E, R289C and R334H) (33). However, this position is not required for the milder effect of these mutations. One selective tooth agenesis-causing EDA mutation, G165A, is located at the end of the collagen-like domain (G. Mues, R. Griggs, A. J. Hartung, G. Whelan, L. G. Best, A. K. Srivastava and R. N. D'Souza, manuscript in preparation); two others (R65G (6) and E67V (12)) are located at the extracellular end of the EDA transmembrane sequence. The R65G and E67V replacements may reduce the expression or translocation, and therefore the dosage of otherwise normal ligands. In HED patients, this area is less frequently affected and the predominance of frameshift and nonsense mutations suggests that the importance of mutations occurring in this part lies more in the abolishment of the rest of the protein than in a local dysfunctional effect. On the other hand, amino acid changes in this least conserved region of EDA are responsible for major adaptive changes in stickleback evolution (27) and may have some other important function.

In our previous functional analysis of HED causing EDA mutations (8), we noticed that the only mutation with residual receptor-binding activity, S374R, came from a family with a predominantly missing teeth phenotype. However, we could not determine if this was a coincidence or if it was a truly tissue-specific phenomenon. Our current results strongly suggest that there is indeed a tissue-specific effect, in that residual EDA/EDAR activity can prevent the hypoplasia of most ectodermal appendages except for the development of incisors and a few other teeth. In other words, the development of teeth, especially of incisors, and premolars requires the highest dosage of EDA signaling. However, the mutations associated with tooth agenesis are remarkably dissimilar in the effects they produce in assays with recombinant EDA. The adjacent mutations V365A and M364T are well expressed and show modestly lower binding to EDAR, which translates into a major impairment at activating a cross-linking-dependent surrogate signaling pathway. It is possible that EDAR signals in teeth are highly sensitive to proper multimerization of EDA whereas EDAR signaling required for hair and gland development may be less stringent in this respect. It will be of interest to test this hypothesis using in vivo approaches.

For S374R, we reproduce the weak binding of the EDA1 isoform to EDAR (8), but see little if any activity in the bioassay. We also observe a much reduced but detectable binding of the EDA2 isoform of this mutant to XEDAR. The mutant proteins T338M and D316G share similar functional properties like S374R with D316G performing worst in the EDAR-binding assay and D316G family members presenting with anodontia or severe oligodontia. Mutation Q358E has different effects. Binding to XEDAR and EDAR is little affected and it retains significant capacity to activate multimerization-dependent signal transduction. However, the 90% reduced secretion level may explain the observed phenotype.

Altogether, we propose that EDA mutations associated with selective tooth agenesis share the property to retain some receptor signaling activity, but to be impaired either in receptor binding, and/or in their expression level and/or in their ability to induce efficient signaling downstream of the receptor. The phenotype strongly suggests that the threshold for EDA signaling in tooth development must be higher than in other EDA-dependent structures. Tooth shape abnormalities appear to be less frequent in EDA-

caused selective tooth agenesis possibly reflecting different requirements for the level of EDA/ EDAR activity in shape formation. There are other examples where different levels of residual gene function can lead to sometimes dramatically different clinical phenotypes: More severe mutations in the X-chromosomal NEMO gene which operates downstream in the EDA/EDAR signaling pathway, lead to incontinentia pigmenti in females and prenatal lethality in males. Milder mutations present as HED with severe immunodeficiency in surviving males (26).

The finding that tooth development requires more EDA pathway signaling than other ectodermal appendages is supported by two observations made in EDA-deficient animals treated with a recombinant form of EDA1 endowed with cross-placental transfer properties by fusion to the Fc portion of IgG1 (Fc-EDA1). When Fc-EDA1 was administered to pregnant EDA-deficient (Tabby) mice, most Tabby-related defects were permanently rescued in offspring, except for tooth number and a special type of murine hair (28). In the second experiment, recombinant EDA preparations were injected postnatally into dog pups with the HED syndrome. It was recognized that the development of the permanent teeth required a relatively higher or repeated dosage of recombinant EDA-A1 (29). These findings also point to the exciting possibility that the isolated tooth agenesis of permanent teeth caused by EDA mutations may be preventable in humans. Health care professionals should be aware of the possibility that patients with agenesis of incisors and various other anterior teeth may carry a mutation in the *EDA* gene without showing other classical ectodermal dysplasia symptoms.

MATERIALS AND METHODS

Patient Recruitment and Phenotype Evaluation
The family participated in our IRB approved tooth agenesis study. After obtaining informed consent, a pedigree was established showing that only males were affected. Phenotype evaluation was performed via a HED- specific questionnaire, interviews and panoramic radiographs of the dentition and/or dental records.

PCR Amplification and Sequencing
Blood samples or buccal swabs were collected for DNA extraction. A candidate gene approach was chosen with the *EDA* gene as a first target. Eight exons of the *EDA* gene were PCR amplified and sequenced with automated fluorescent dideoxy technology.

Testing Family and General Population
A T to C transition in the last exon of the*EDA* gene of the affected index male was found to create a HhaI restriction site. All other participating members of the family as well as sixty-five unrelated females and 14 unrelated males were tested for the presence of this restriction site. Primers 5'CACGCCTTCACATGGCACT3' and 5'CGGCTGCAACACCAATACAC3' were used for amplification of the exon.

Preparation of EDA Expression Vectors
Mammalian expression vectors for secreted Flag-tagged forms of EDA1 and EDA2 with the tooth agenesis-causing mutations V365A, Q358E, D316G, T338M or M364T were generated as described previously for other EDA mutants including S374R (8). Briefly, these constructs code for the signal peptide of haemagglutinin, the Flag sequence (DYKDDDDK), a linker (GPGQVQLQVD) and the TNF homology domain of EDA1 (amino acids 245-391) or EDA2 (amino acids 245-389). Other EDA constructs used in this study and expression vectors for human EDAR-Fc and human XEDAR-Fc have been described previously (8).

Expression of Soluble EDA in Cell Supernatants
Vectors encoding soluble, wild type (wt) or mutant Flag-tagged EDA1 or EDA2 protein, or EDAR:Fc were transfected into 293T cells and cells were maintained in serum-free OptiMEM for 7 days. The

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concentration of Flag-tagged EDA proteins in cell supernatants was estimated by comparative anti-Flag Western blot using serial dilutions of each protein and known amounts of purified Flag-TRAIL.

Efficiency of EDA secretion

   293T cells were transfected with wild type or mutant Flag-EDA1, and supernatant and cells were harvested separately. The cell pellets were lysed by sonication in a known volume of SDS-PAGE sample buffer. 2-fold serial dilutions of cell extracts and the corresponding supernatants were evaluated for relative EDA protein content by densitometry of slot blots developed with anti-Flag. Percent secretion was calculated as (EDA amount in supernatant) divided by (EDA amount in cells + supernatant) times 100.

ELISA Assay for Receptor-Ligand Binding

   The binding of wild type and mutant EDA1 and EDA2 to EDAR and XEDAR was monitored by ELISA as previously described (8, 30). Briefly, ELISA plates were coated with a mouse anti-human monoclonal antibody (100 µl at 5 µg/ml in 50 mM Na-Carbonate pH9), blocked, incubated with 100 µl of EDAR:Fc or XEDAR:Fc (Alexis, Lausen, Switzerland) at 0.5 µg/ml, followed by two-fold dilutions of quantified Flag-EDA. Bound ligands were revealed with biotinylated anti-Flag (0.5 µg/ml. Sigma), horse radish peroxidase-conjugated streptavidin (1/4000. Jackson Immunoresearch), and orthophenylene diamine (OPD) reagent, and read at 492 nm.

Generation of EDAR:Fas Jurkat Cells

   Fas-deficient Jurkat-JOM2 cells expressing the EDARFas fusion protein were generated essentially as described previously for BCMA:Fas (31). For this purpose, the retroviral vector pMSCVpuro encoding the extracellular domain of human EDAR (amino acids 1-183), amino acids VD and the transmembrane and intracellular domains of human Fas (amino acids 169-335) was utilized. About 40 clones were tested for their sensitivity to Fc-EDA1 (28), and one of the sensitive clones (Jurkat-2199 clone 23) was selected for further experimentation. In these cells, binding of EDA1 to EDAR: Fas induces cell death through the Fas signaling pathway. Cytotoxicity assays with Flag-EDA1 wt or mutants were performed in the presence or absence of 2 µg/ml of cross-linking anti-Flag M2 monoclonal antibody (Sigma) as previously described (23, 32).

CONFLICT OF INTEREST

We declare no conflict of interest.

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FIGURE LEGENDS
Figure 1. Pedigree and phenotype of a family with selective tooth agenesis.
A. Pedigree of the family with non-syndromic tooth agenesis and segregation of the Hha1 restriction site (created by the T to C transition in the EDA gene) with affected and carrier status. Numbers 1-26 denote the family members for whom DNA samples were available. Open circles: female, open squares: male, filled squares: affected male , / deceased. B. Tooth agenesis pattern in affected males. Incisors are most frequently absent, in particular mandibular incisors. Maxillary central incisors are rarely affected in this Caucasian family, consistent with previous reports (25).
Primary teeth: 1= central incisors, 2= lateral incisors, 3=canines, 4-5=molars; permanent teeth: 1= central incisors, 2= lateral incisors, 3= canines, 4-5= premolars; 6-8= molars.

Figure 2. Amino acid comparison of EDA proteins from different species and position of the tooth agenesis-causing mutations
A. Alignment of a portion of the TNF homology domain of EDA from different species, showing the locations of V365A and five further missense mutations causing isolated tooth agenesis (D316G, T338M, Q358E, M364Tand S374R). The EDA amino acid sequences for human, chimp, rhesus, cow, dog, rat and mouse are identical in this region and are referred to as "mammals". Numbering corresponds to the human sequence. The position of some HED-causing mutations and of the only known polymorphism are also shown. B. Tooth agenesis-causing EDA mutations are surface-located in the predicted receptor-binding region at the interface of two protomers. Figure is based on the PDB atomic coordinate file 1RJ7.

Figure 3. A. Normalized amounts of wild-type and mutant Flag-EDA1 (upper panel) or Flag-EDA2 proteins (lower panel) from supernatants of transfected 293T cells were analyzed by anti-Flag Western-blot. B. Flag-EDA1 (wild type and the indicated mutants), Flag-EDA2 (wild type) and the positive control Flag-TWEAK were treated with or without peptide-N-glycanase F (PNGaseF) and analyzed by anti-Flag western blot. C. Similar amounts of Flag-EDA1 wt and V365A were analyzed by anti-Flag Western blot to validate the estimation of protein concentration. D. Flag-EDA1 present in cell extracts and cell supernatants were quantified in order to estimate secretion efficiency. All constructs contain the

signal peptide of hemaglutinin. Protein secretion is consistently impaired by the tooth agenesis-causing mutation Q358E and the HED-causing mutation Y343C.

Figure 4. A. Quantitative binding of EDA1 to EDAR (upper panel) and EDA2 to XEDAR (lower panel), as measured by ELISA. All six tooth agenesis-causing mutants retain residual receptor-binding to EDAR in contrast to the HED-causing mutants Y343C and H252L which loose all receptor-binding ability. Binding of EDA2 to XEDAR is not or only moderately affected for five of the six tooth agenesis-causing mutants. The results are representative of 3 to 6 experiments (depending on the mutant) with similar results. B. EDAR:Fas-expressing Jurkat cells were treated with the indicated concentrations of wt and mutant EDA1 preparations. The surrogate apoptotic Fas pathway is monitored as reduced cell viability using the PMS/MTS reagents. Successful activation occurs only in the presence of a cross-linking anti-Flag antibody. Only wild type EDA1 and mutant Q358E were significantly active in this assay. The results are representative of three independent experiments with similar results.
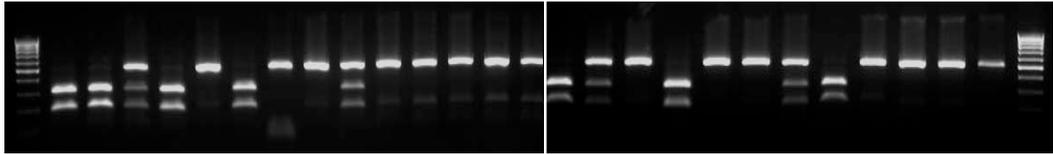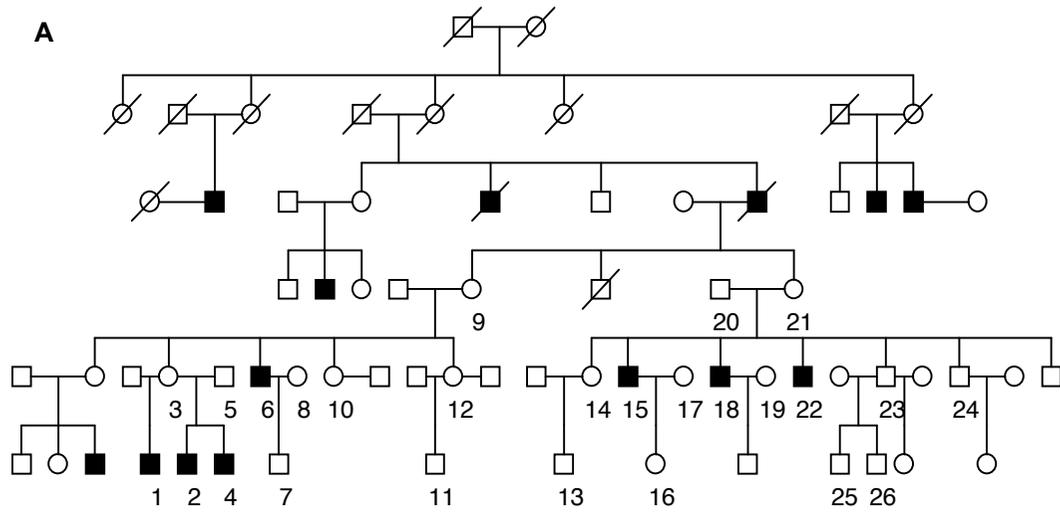
Table 1: Frequency of HED-like symptoms in 7 affected males, 5 carrier females and 8 unaffected family members. These symptoms have been rated as "clearly present or frequently a problem".

	Affected (7)	Carrier (5)	Unaffected (8)
Spares, thin hair	1/7	0/5	0/8
Early balding*	1/4	0/5	0/8
Thin skin	1/7	0/5	0/8
Overheating easily	2/7	1/5	3/8
High childhood fevers	0/7	1/5	2/8

* only adults are counted.

ABBREVIATIONS

The abbreviations used are: AXIN2, Axis Inhibitor 2 gene/ protein; EDA, ectodermal dysplasia A gene/ protein; EDAR, EDA1 receptor; HED, hypohidrotic ectodermal dysplasia syndrome; MSX1, Muscle Segment Homeobox 1 gene/ protein; PAX9, Paired Box 9 gene/ protein; TNF, Tumor Necrosis Factor; RFLP, restriction fragment length polymorphism; WNT, wingless-type MMTV integration site family member 1 gene/protein; XEDAR, X-linked EDA2 receptor.
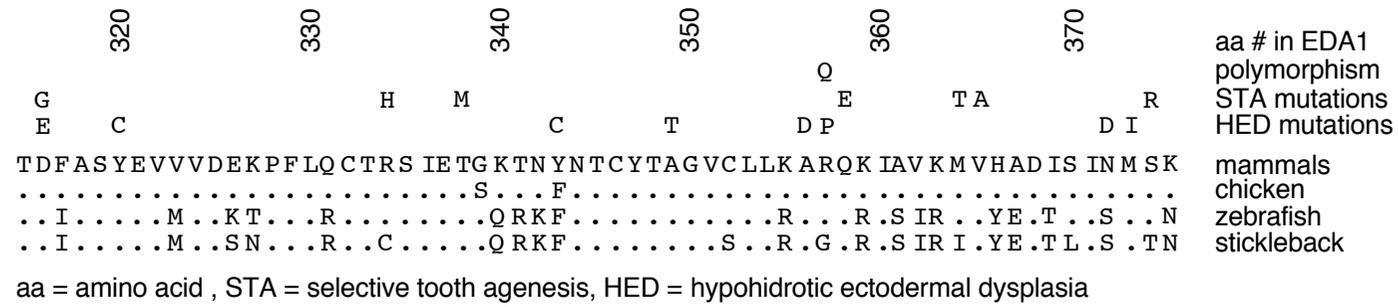
Mues et al, Figure 1

A

320	330	340	350	360	370	aa # in EDA1

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                                           Q                        polymorphism
  G                 H    M                   E         T A          R    STA mutations
  E     C                        C         T      D P                 D I  HED mutations

TDFASYEVVVDEKPFLQCTRS IETGKTNYNTCYTAGVCLLKARQK IAVKMVHADIS INMSK   mammals
........................S...F.............................   chicken
..I.....M..KT...R........QRKF.............R...R.SIR..YE.T..S..N   zebrafish
..I.....M..SN...R..C.....QRKF.........S..R.G.R.SIRI.YE.TL.S.TN   stickleback
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aa = amino acid , STA = selective tooth agenesis, HED = hypohidrotic ectodermal dysplasia
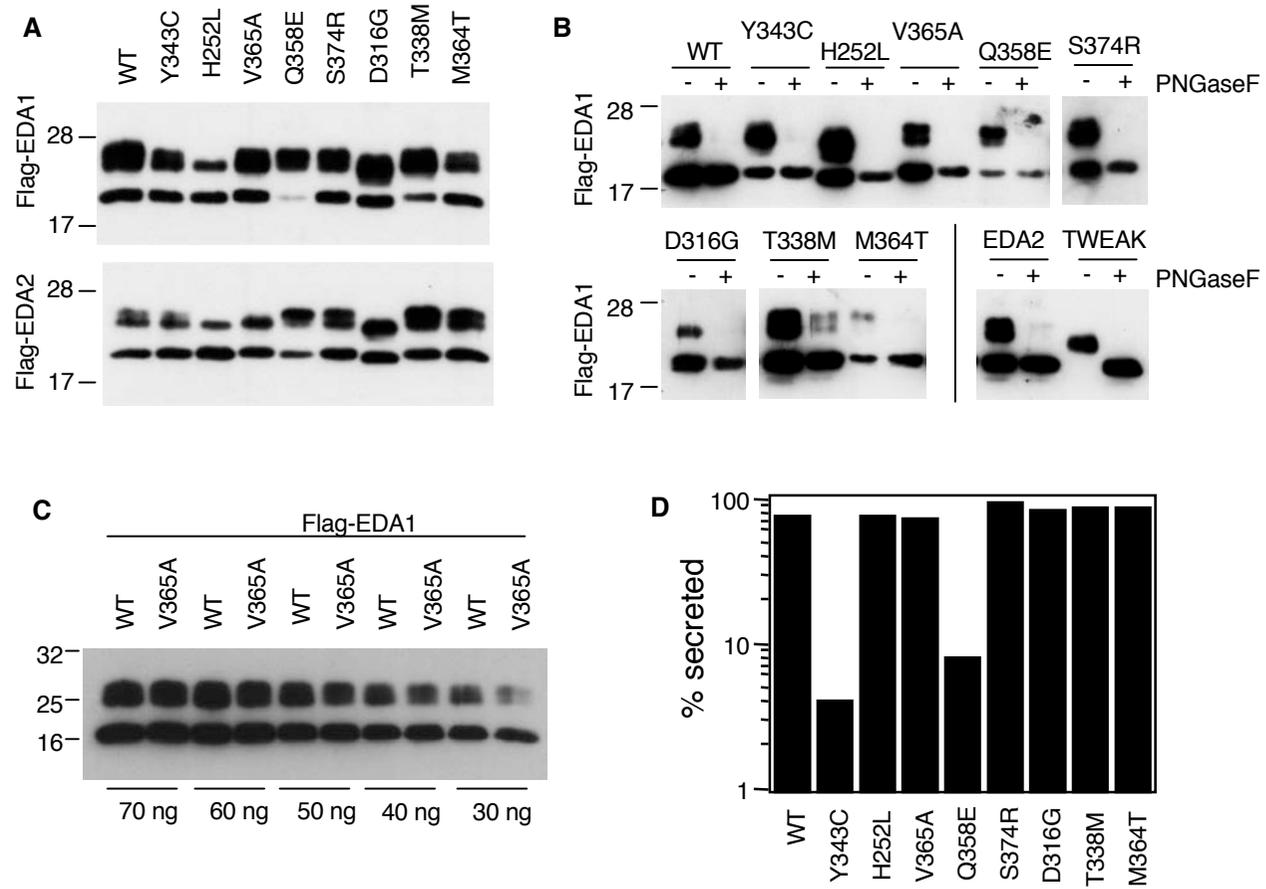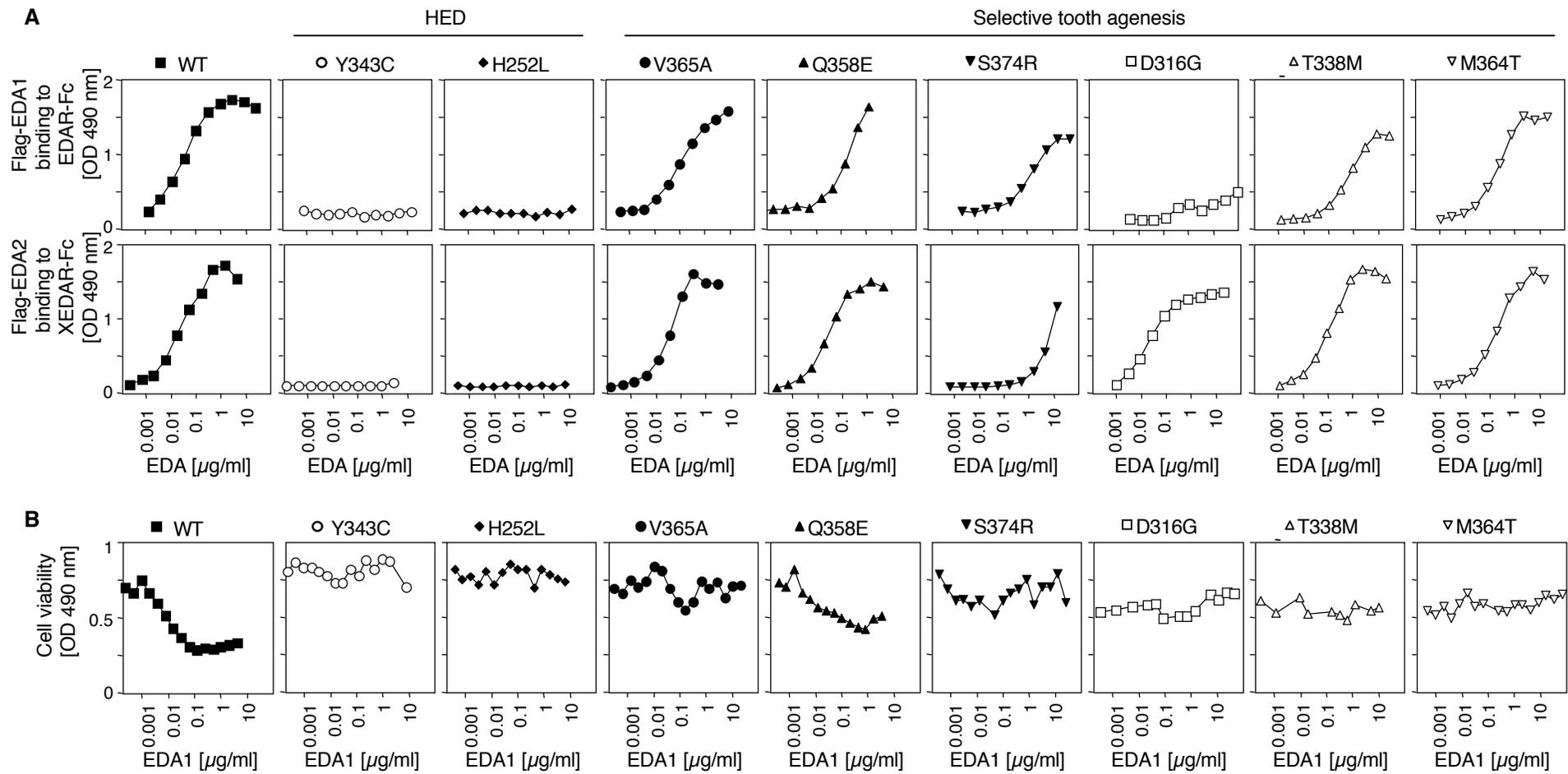
B

Q358

S374

M364

V365

D316

T338

Mues et al, Figure 2

Mues et al, Figure 3

Mues et al, Figure 4