Heterozygous Loss-of-Function Mutations in *DLL4* Cause Adams-Oliver Syndrome

Report

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Abstract

Adams-Oliver syndrome (AOS) is a rare developmental disorder characterized by the presence of aplasia cutis congenita (ACC) of the scalp vertex and terminal limb reduction defects. Cardiovascular anomalies are also frequently observed. Mutations in five genes have been identified as a cause for AOS prior to this report. Mutations in *EOGT* and *DOCK6* cause autosomal recessive AOS, whereas mutations in *ARHGAP31*, *RBPJ* and *NOTCH1* lead to autosomal dominant AOS. As *RBPJ*, *NOTCH1* and *EOGT* are involved in NOTCH signaling, we hypothesized that mutations in other genes involved in this pathway may also be implicated in AOS pathogenesis. Using a candidate gene based approach, we prioritized DLL4, a critical NOTCH ligand, due to its essential role in vascular development in the context of cardiovascular features in AOS individuals. Targeted resequencing of the *DLL4* gene using a custom enrichment panel in 89 independent families resulted in the identification of 7 mutations. A defect in *DLL4* was also detected in two pedigrees with whole exome/genome sequencing. In total, nine heterozygous mutations in *DLL4* were identified, including two nonsense and seven missense variants, the latter encompassing four mutations that replace or create cysteine residues, which are likely critical for maintaining structural integrity of the protein. Affected individuals with *DLL4* mutations present with variable clinical expression with no emerging genotype-phenotype correlations. Our findings demonstrate that *DLL4* mutations are an additional cause of autosomal dominant AOS or isolated ACC and provide further evidence for a key role of NOTCH signaling in the etiology of this disorder.

Adams-Oliver syndrome (AOS [MIM100300]) is a rare developmental disorder with an estimated incidence of 1 in 225,000 live births.[1] It is typically characterized by the presence of both aplasia cutis congenita (ACC) of the scalp vertex and terminal limb defects, such as brachydactyly, oligodactyly, syndactyly, hypoplastic nails or transverse amputations.[2] In addition, vascular and cardiac anomalies, comprising pulmonary hypertension, ventricular septum defects, tetralogy of Fallot and anomalies of the great arteries and their valves are frequently observed.[2] In the past few years, mutations in five genes have been described as a cause of AOS. Mutations in *EOGT* (MIM 615297) and *DOCK6* (MIM 614219) cause the autosomal recessive form of AOS, while mutations in *ARHGAP31* (MIM 100300), *RBPJ* (MIM 614814) and *NOTCH1* (MIM 616028) lead to the autosomal dominant form of AOS.[3-8] *NOTCH1, EOGT,* and *RBPJ* are all members of the NOTCH signaling pathway. This pathway is conserved throughout metazoan species and involved in many different tissue specific cellular processes, including cell-fate determination and neural and hematopoietic stem cell differentiation.[9; 10] The NOTCH receptors and ligands are composed of several domains, including epidermal growth factor (EGF)-like domains. *EOGT* is an EGF domain-specific O-linked N-acetylglucosamine (GlcNAc) transferase known to glycosylate NOTCH1 and is therefore highly likely to influence NOTCH signaling through post-translational modification of the extracellular EGF-like repeats.[4; 10; 11] RBPJ is a sequence specific transcription factor that regulates the transcriptional activity of downstream target genes by binding to the intracellular domain of NOTCH, leading to the recruitment of additional proteins.[12] Besides AOS, mutations in *NOTCH1* are also known to cause bicuspid aortic valve and thoracic aortic aneurysm.[13] NOTCH signaling is activated by two families of ligands, namely Jagged (JAG1 or -2) and Delta (DLL1, -3 or -4). Of these, DLL4 has an essential role in vascular development and angiogenesis, which places it as a prime candidate for AOS, due to the presence of cardiovascular features in AOS individuals.[14; 15]

The study was approved by the appropriate institutional review board (IRB) and appropriate informed consents were obtained from human subjects. We performed targeted resequencing of

DLL4 on 89 individuals using HaloPlex Targeted Enrichment (Agilent Technologies) followed by sequencing on MiSeq (Illumina) using 2 x 150 bp paired end reads. An in-house developed Galaxy-based pipeline was used to process the raw data.[16] Variant calling was performed using the GATK Unified Genotyper[17] and variants were annotated and filtered using the in-house developed database, VariantDB.[18] Unique variants (ESP6500 and 1000 Genomes project) were selected, prioritizing nonsense, nonsynonymous, splice site, and indel variants in both a dominant and a recessive model.

Separately, whole genome sequencing (WGS) was performed on family 2 and whole exome sequencing (WES) was performed on family 6. Genomic DNA was extracted from saliva from the two affected siblings of family 2, and subjected to the Agilent SureSelect Target Enrichment (v4; 51 Mb) capture process. Following library preparation, sequencing was performed on the Illumina HiSeq2000, generating 100 bp paired end reads. Over 97.5% of target regions were covered by >10X reads. Reads were mapped against reference genome UCSC hg19 with BWA. Single nucleotide variants and indels were detected by SAMTOOLS. WES on family 6 was performed as previously described.[7] All variants were confirmed by bidirectional Sanger sequencing and segregation analysis was performed on available family members.

In total, we screened 91 families and identified nine heterozygous variants in *DLL4* (RefSeq transcript NM_019074.3, with GRCh37 as a reference build), including nonsense, cysteine replacing or creating and other missense substitutions (Figure 1, Table S1). These results designate mutations in this gene as an additional cause of an autosomal dominant form of AOS.

In two families, we detected nonsense mutations in exon 9 of *DLL4*, specifically c.1660C>T (p.Gln554*; ClinVar accession number SCV000240088) in family 1 and c.1672C>T (p.Arg558*; ClinVar SCV000240089) in family 2. Both stopgain mutations are predicted to lead to nonsense mediated decay (NMD) of the mutant mRNA transcript (Figure 1B).[19] The severity of the clinical features in the affected family members of both families varied widely and included ACC, syndactyly and

brachydactyly (Figure 2, Table 1). In the proband of family 1 (1-III-3), cardiovascular features were also present, namely tricuspid insufficiency and ventricular septum defect (Table 1). Molecular screening of available family members of family 1 showed segregation of the mutation in the affected sister (1-III-1) and mother (1-II-2), and absence of the mutation from the unaffected father (1-II-1) and maternal grandmother (1-I-2). Interestingly, the mutation was not detected in the maternal grandfather (1-I-1), who presented with unilateral brachydactyly of the toes (III and IV). We failed to demonstrate the possibility of somatic mosaicism in his blood by a mutation allele specific PCR. Nevertheless, somatic mosaicism not affecting the hematopoietic tissue remains a plausible explanation. In family 2, the affected sister (2-II-1) also carried the mutation. Mutation analysis of the parents showed that c.1672C>T was inherited from their father (2-I-1) who did not show obvious signs of AOS. DNA of the paternal grandparents was not available to investigate whether the mutation in the father occurred *de novo*.

In addition to the two stopgain mutations, we identified seven different heterozygous missense mutations: three cysteine replacing mutations, one cysteine creating mutation and three other missense mutations. All identified missense mutations were predicted to be damaging by MutationTaster, PolyPhen-2 and SIFT and none of the mutations were present in the following public databases: ESP6500, Kaviar,[20] 1000 Genomes Project and ExAC database.

In families 3, 4 and 5, cysteine replacing mutations (c.1365C>G, p.Cys455Trp [ClinVar SCV000240090]; c.1169G>A, p.Cys390Tyr [ClinVar SCV000240091]; c.1168T>C, p.Cys390Arg [ClinVar SCV000240092], respectively) in exons 8 and 9 of *DLL4* were identified. All of these mutations affect critical and highly conserved (up to D. melanogaster) cysteines of the consensus sequence of either the 5th or 7th EGF-like domain (Figure 1C). Similar to the nonsense mutations, the severity of the clinical features of all available affected family members varied greatly, but all showed scalp involvement, including ACC or a bald scalp area (Table 1, Figure 2). The c.1365C>G (p.Cys455Trp) mutation, identified in the proband of family 3 (3-II-1), was confirmed in the affected mother (3-I-2) and in a sister (3-II-2), but no clinical information of the latter was available. In both family 4

(c.1169G>A, p.Cys390Tyr) and family 5 (c.1168T>C, p.Cys390Arg), we found a mutation replacing the cysteine residue at position 390 but leading to different amino acid substitutions. The proband of family 4 (4-II-1) showed isolated ACC and no family members were available for screening. The proband of family 5 (5-IV-3) is part of a large family with multiple affected individuals. The father (5-III-3) and two great aunts (5-II-14, 5-II-17) carried the c.1168T>C mutation (Figure 1A). They were all only mildly affected, but did have severely affected offspring (Table 1, Figure 2), further illustrating the highly variable clinical expression for *DLL4* associated AOS.

In the proband of family 6 (6-II-1) we detected a heterozygous cysteine creating mutation: c.556C>T (p.Arg186Cys; ClinVar SCV000240093). The affected family members both exhibited isolated ACC (Table 1, Figure 2). The arginine at this position is conserved between DLL4 and DLL3 and is conserved across mammalian species (Figure 1C). The affected father (6-I-1) carried the mutation, while the unaffected mother (6-I-2) did not.

In families 7, 8 and 9, we found missense variants (c.799C>A, p.Pro267Thr [ClinVar SCV000240094]; c.361G>C, p.Ala121Pro [ClinVar SCV000240095]; c.583T>C, p.Phe195Leu [ClinVar SCV000240096], respectively) affecting specific residues in functional domains of DLL4 which are conserved up to D. melanogaster and in the four most closely related ligands of the NOTCH receptors, namely JAG1, JAG2, DLL1 and DLL4 (Figure 1C). Both families 7 and 8 presented with scalp defects, limb defects and cardiovascular features (Table 1, Figure 2). The parents of proband 7 were reported as unaffected, but the mother (7-I-2), who was a mutation carrier, has a positive family history for cardiac events with a brother with bicuspid aortic valve with narrowed aorta ascendens and a maternal half-brother who died at birth of an unspecified heart defect. Unfortunately, DNA of the maternal grandparents or other family members was not available. In the proband of family 8 (8-II-1) the c.361G>C (p.Ala121Pro) mutation was shown to be *de novo*, as both unaffected parents did not carry the mutation (Figure 1A). The index of family 9 has been described before and shows only aplasia cutis congenita.[21] In this individual we identified a heterozygous c.583T>C (p.Phe195Leu) variant. No further information of familial occurrence was available.

The DLL4 variants are distributed across the complete protein, affecting all known structural domains (MNNL [N-terminal domain of NOTCH ligands], DSL [Delta/Serrate/Lag-2] and EGF-like domains), without any obvious mutational hotspots. Recently, the structural basis of the interaction between NOTCH1 and DLL4 has been elaborated. [10] The binding of NOTCH1 to DLL4 is coordinated by the interaction between the NOTCH1 EGF-like 11 and the DLL4 DSL domain. DLL4-NOTCH1 binding is further modulated by the interaction between NOTCH1 EGF-like 12 and the MNNL domain of DLL4. Glycosylation of NOTCH1 is essential and specific for the binding of DLL4.[10]

Three-dimensional modeling predictions of our DLL4 mutations reveals interesting changes in protein conformation and amino acid functionality (Figure S1). In total, three cysteine replacing mutations have been found, two at position 390 (p.Cys390Arg and p.Cys390Tyr), located in EGF-like domain 5 and one at position 455 (p.Cys455Trp), located in the 7th EGF-like domain. All three cysteines are involved in the formation of a disulfide bond in their respective EGF-domain, which will be lost due to the introduction of these mutations. Furthermore, both arginine and tyrosine residues at position 390 are too bulky to fit in this domain and are therefore likely to disrupt the structure. Hence, structural changes are predicted to occur, which will most likely lead to a loss of function of DLL4.

Both phenylalanine at position 195 and arginine at position 186 are located in the DSL domain of DLL4 (Figure 1B), a conserved central domain directly involved in ligand binding to the 11th EGF-like domain of the NOTCH1 receptor.[10] It has been shown that the phenylalanine at position 195 is a key interface residue important for binding and when mutated to an alanine residue it results in a substantial decrease in NOTCH1 interaction.[10] The p.Arg186Cys mutation is located in the surface loop of the DSL domain. The introduction of a cysteine at this location is not expected to cause steric hindrance; however, the important interaction function of the Arginine186 residue is likely to be lost. In addition, this mutation creates a cysteine, which could dramatically alter the conformation of the protein by forming a novel disulfide bond. Both these DSL domain mutations will likely reduce the binding affinity of DLL4 to NOTCH1.

The Alanine121 residue is located in a beta-strand on the inside of the MNNL domain. Together with the DSL domain, the MNNL domain is involved in the binding of the ligand to EGF-like domain 12 of the NOTCH-receptor and regulates ligand pleiotropy.[10] The replacement of Alanine 121 by proline is predicted to disrupt the local structure and function. Lastly, the Proline267 residue is located in EGF-like domain 2 and makes contact with EGF-like domain 1 in a surface loop. This interaction might be altered by the substitution to threonine.

The discovery of *DLL4* mutations in our AOS individuals in addition to prior identification of mutations in *EOGT*, *RBPJ* and *NOTCH1*, confirms the key role of the NOTCH signaling pathway in the pathogenesis of AOS. Activation of NOTCH signaling through binding of a NOTCH-ligand (JAG1, JAG2, DLL1, DLL3, DLL4) to one of the NOTCH receptors (1 to 4), results in two proteolytic cleavage reactions performed by a member of the ADAM (A Disintegrin and metalloproteinase domain-containing protein) family and by the γ-secretase complex. The second cleavage releases the intracellular domain of NOTCH (NICD), which is translocated to the nucleus, where it binds to the coactivator Mastermind Ligand (MAML) and the DNA binding protein RBPJ to induce transcription of NOTCH target genes.[22]

DLL4 is a ligand of the NOTCH receptors and is essential for vascular development.[12] During embryonic development, protein localization of DLL4 is restricted to the large arteries, while in adult stages protein levels are found in the smaller arteries and microvessels. Haploinsufficiency of Dll4 in mice results in embryonic lethality due to profound defects in vascular and arterial development.[15] In contrast to mice models, human haploinsufficiency of DLL4 does not result in lethality as we have identified two heterozygous nonsense mutations, which most likely lead to NMD.[19] The lethality in mice could be related to the inbred genetic background of the mice[23] and potential functional differences in redundancy between humans and mice. The identification of both *DLL4* nonsense mutations and several missense mutations in key functional domains of DLL4 that are predicted to disrupt DLL4 integrity, suggests that loss-of-function is the pathogenetic mechanism. While DLL4 is

described to be essential for vascular development[12], not all individuals with DLL4 mutations presented with cardiovascular features (Table 1), although complete cardiovascular exams have not been performed in all cases.

We did not observe a clear genotype-phenotype correlation between *DLL4* mutation and AOS subtype. Furthermore, we observed marked intrafamilial variability in the phenotypic expression. For example, in family 5 the p.Cys390Arg substitution leads to a severe phenotype in the offspring, while the parents show isolated ACC. This suggests that other factors are involved in the clinical expression of this disease, which could be environmental factors, but also other genetic and epigenetic influences involved in the NOTCH signaling pathway or other AOS related pathways. In addition to the variable clinical expression, we also observed incomplete penetrance in families 2 and 7, and additional unidentified asymptomatic mutation carriers may exist in other families. Highly variable expressivity and incomplete penetrance, as well as the association with cardiovascular abnormalities are similar to what has been observed in *NOTCH1*-related AOS.[7; 8] In contrast, cerebral and ocular abnormalities which have been found to be particularly associated with autosomal recessive DOCK6 related AOS appear to be uncommon in those autosomal dominant forms. [24]

In conclusion, through screening a cohort of 91 families affected with Adams-Oliver syndrome or aplasia cutis congenita, we have identified nine heterozygous variants in *DLL4*, including nonsense, cysteine replacing or creating and other missense mutations, demonstrating that mutations in this gene are an important cause of autosomal dominant AOS or isolated ACC. Affected individuals show variable clinical expression with at present no clear genotype-phenotype correlations. With the addition of *DLL4*, four genes involved in NOTCH signaling have now been implicated in AOS, confirming that disruption of this pathway plays a major role in the pathogenesis of AOS.

Supplemental data

Supplemental data include one figure and one table.

Acknowledgments

We are grateful to the families who participated in this study. B.L.L. is a senior clinical investigator of the Fund for Scientific Research, Flanders (FWO, Belgium) and holds an ERC starting grant. J.A.N.M. is a predoctoral researcher of the Fund for Scientific Research, Flanders (FWO, Belgium). ABS was supported by the German Academic Exchange Service (DAAD) and the German Research Foundation (DFG). This research was supported by funding from the Fund for Scientific Research, Flanders (FWO, Belgium) [G.0221.12], the Fondation Leducq, and the European Research Council (ERC), a Center for Systems Biology P50 grant (GM076547, US National Institutes of Health) and the Rare Disease Foundation.

Web Recourses

1000 Genomes Project, http://www.1000genomes.org/

ExAC Browser, http://exac.broadinstitute.org/

Mutationtaster, http://www.mutationtaster.org/

NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.jcvi.org/

UCSC genome browser (hg19), http://genome.ucsc.edu/

BWA, http://bio-bwa.sourceforge.net/

SAMTOOLS, http://samtools.sourceforge.net/

Accession Numbers

The ClinVar accession numbers for the variants reported in this paper are SCV000240088, SCV000240089, SCV000240090, SCV000240091, SCV000240092, SCV000240093, SCV000240094, SCV000240095 and SCV00024096.

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Table 1 Clinical features

Individual	Internal reference	Mutation status	Clinical features			
			Scalp defects	Limb defects	Cardiovascular features	Other remarks
1-I-1	SK039	-	/	Left brachydactyly of 3rd and 4th toe (confirmed by X-ray)	/	/
1-II-2	SK039	+	/	Short distal phalanges (not confirmed by X-ray)	/	/
1-III-1	SK039	+	ACC	/	Normal echocardiogram	/
1-III-3	SK039	+	ACC	Brachydactyly, syndactyly of 2nd and 3rd toe on right foot	Tricuspid insufficiency, ventricular septum defect	/
2-II-1	F45	+	ACC with underlying skull defect	Brachydactyly left foot; missing toes right foot	Normal echocardiogram	/
2-II-3	F45	+	ACC with underlying skull defect	Brachysyndactyly right foot; severe brachysyndactyly left foot	Normal echocardiogram	Small kidneys; mild hypertension
3-I-2	SK038	+	ACC	/	/	/
3-II-1	SK038	+	ACC	/	/	/
4-II-1	SK049	+	ACC	/	/	/
5-II-4	SK087	No DNA	ACC	Syndactyly of 2nd and 3rd toe, hypoplastic toe nails, brachydactyly of toes	/	/
5-II-14	SK087	+	Bald area on scalp	/	/	Cutis marmorata
5-II-17	SK087	+	Bald area on scalp	Brachydactyly of fingers and toes, syndactyly of 2nd and 3rd toe	/	/
5-III-3	SK087	+	Bald area on scalp	/	/	/
5-III-21	SK087	No DNA	Bald area on scalp	/	/	/
5-III-23	SK087	No DNA	ACC with underlying skull defect	/	/	/
5-IV-3	SK087	+	ACC	/	Normal echocardiogram	Cutis marmorata, epilepsy, learning difficulties, borderline intellectual function (TIQ 76) at young age, normal IQ at later age, mild periventricular leukomalacia
5-IV-12	SK087	No DNA	ACC	/	Normal echocardiogram, portal hypertension, esophageal varices	Splenomegaly, congenital liver fibrosis
6-I-1	UK16	+	ACC	/	No cardiac problems	/
6-II-1	UK16	+	ACC	/	Normal echocardiogram	/
7-I-2	SK082	+	/	/	/	Unaffected
7-II-1	SK082	+	ACC, delayed ossification	Hypoplastic toe nails	No cardiac problems	Cutis marmorata, normal chest X-ray at age two months
8-II-1	SK081	+	ACC	Short distal phalangus of middle finger of the right hand, symphalangism of the index finger, symbrachydactyly of both feet (front and middle foot missing on right foot)	Truncus arteriosus, ventricular septum defect	Growth hormone deficiency
9-II-1	SK013	+	ACC	/	/	/

The following abbreviations are used: ACC, Aplasia Cutis Congenita; /, unknown

Figure 1. Mutation analysis

(A) Pedigrees of the families with their respective mutation and nucleotide sequence.

(B) Structure of *DLL4* with the structural domains and the identified mutations.

(C) Conservation of specific residues amongst species and NOTCH ligands.

Figure 2. Clinical features

(A) Individual 2-II-1 with brachydactyly of left foot and missing toes right foot.

(B) Individual 5-II-17 with a bald area on the scalp.

(C) Individual 5-II-17 with brachydactyly of toes.

(D) Individual 5-II-17 with brachydactyly of fingers.

(E) Individual 5-IV-12 with aplasia cutis congenita.

(F) Individual 6-II-1 with aplasia cutis congenita.

(G) Individual 8-II-1 with short distal phalangus of middle finger and symphalangism of the index finger of the right hand.

(H) Individual 8-II-1 with aplasia cutis congenita.

(I) Individual 8-II-1 with symbrachydactyly of both feet (front and middle foot missing on right foot).

Figure 1.

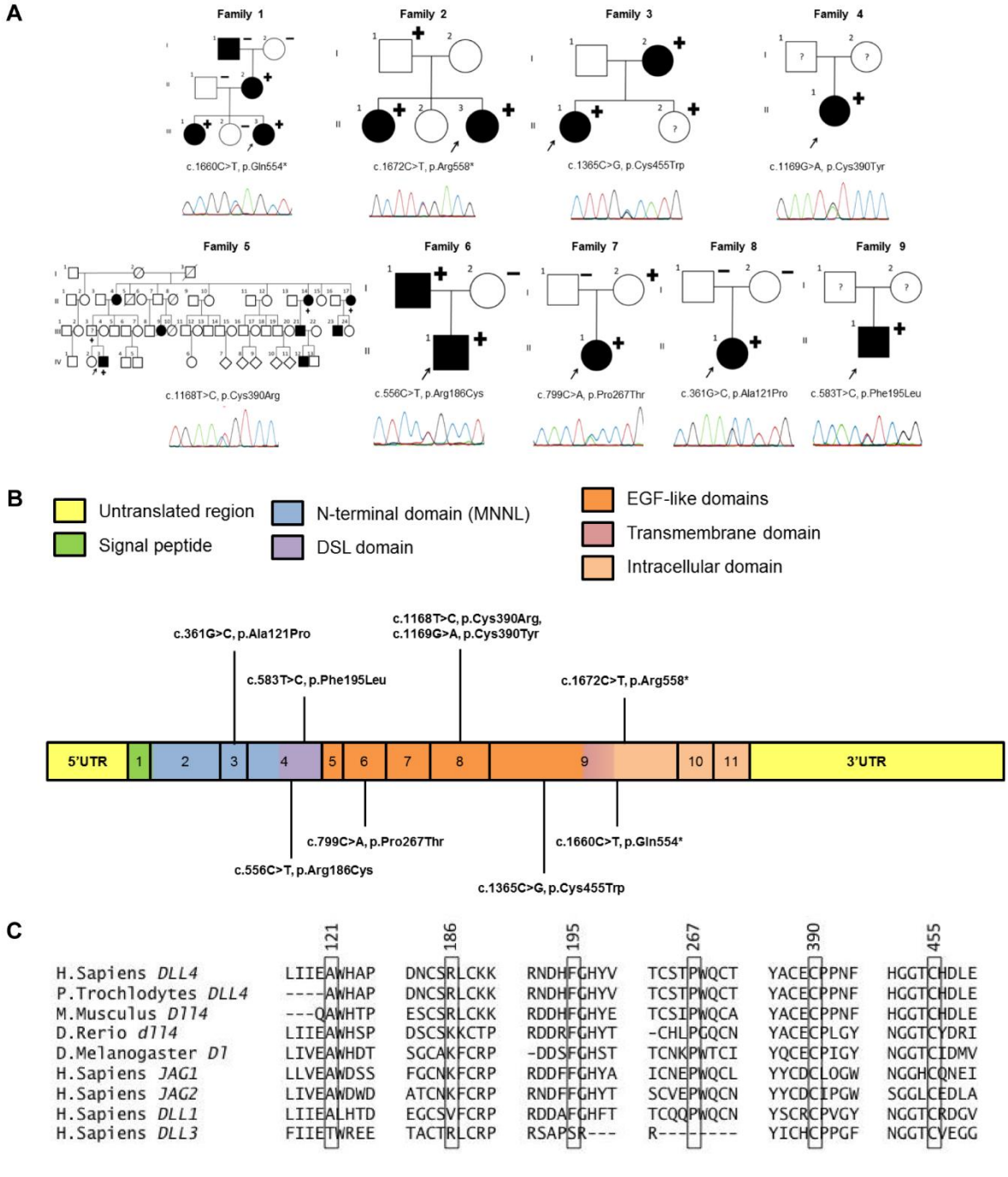
A
Family 1
Family 2
Family 3
Family 4
c.1660C>T, p.Gln554*
c.1672C>T, p.Arg558*
c.1365C>G, p.Cys455Trp
c.1169G>A, p.Cys390Tyr
Family 5
Family 6
Family 7
Family 8
Family 9
c.1168T>C, p.Cys390Arg
c.556C>T, p.Arg186Cys
c.799C>A, p.Pro267Thr
c.361G>C, p.Ala121Pro
c.583T>C, p.Phe195Leu
B
Untranslated region
Signal peptide
N-terminal domain (MNNL)
DSL domain
EGF-like domains
Transmembrane domain
Intracellular domain
c.361G>C, p.Ala121Pro
c.583T>C, p.Phe195Leu
c.1168T>C, p.Cys390Arg,
c.1169G>A, p.Cys390Tyr
c.1672C>T, p.Arg558*
5'UTR
1
2
3
4
5
6
7
8
9
10
11
3'UTR
c.556C>T, p.Arg186Cys
c.799C>A, p.Pro267Thr
c.1365C>G, p.Cys455Trp
c.1660C>T, p.Gln554*
C
121
186
195
267
390
455
H.Sapiens DLL4 LIIEAWHAP DNCSRLCKK RNDHFGHYV TCSTPWQCT YACECPPNF HGGTCHDLE
P.Trochlodytes DLL4 ----AWHAP DNCSRLCKK RNDHFGHYV TCSTPWQCT YACECPPNF HGGTCHDLE
M.Musculus D114 ---QAWHTP ESCSRLCKK RDDHFGHYE TCSIPWQCA YACECPPNF HGGTCHDLE
D.Rerio d114 LIIEAWHSP DSCSKKCTP RDDRFGHYT -CHLPGQCN YACECPLGY NGGTCYDRI
D.Melanogaster D1 LIVEAWHDT SGCAKFCRP -DDSFGHST TCNKPWTCI YQCECPIGY NGGTCIDMV
H.Sapiens JAG1 LLVEAWDSS FGCNKFCRP RDDFFGHYA ICNEPWQCL YYCDCLOGW NGGHCQNEI
H.Sapiens JAG2 LIVEAWDWD ATCNKFCRP RNDFFGHYT SCVEPWQCN YYCDCIPGW SGGLCEDLA
H.Sapiens DLL1 LIIEALHTD EGCSVFCRP RDDAFGHFT TCQQPWQCN YSCRCPVGY NGGTCRDGV
H.Sapiens DLL3 FIIETWREE TACTRLCRP RSAPSR--- R--------- YICHCPPGF NGGTCVEGG

Figure 2.