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BRIEF REPORT

DOCK6 Mutations are Responsible for a Distinct Autosomal Recessive Variant of Adams-Oliver Syndrome Associated with Brain and Eye Anomalies

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Abstract

Adams-Oliver syndrome (AOS) is characterized by the association of aplasia cutis congenita with terminal transverse limb defects, often accompanied by additional cardiovascular or neurological features. Both autosomal dominant and recessive disease transmission have been observed, with recent gene discoveries indicating extensive genetic heterogeneity. Mutations of the *DOCK6* gene were first described in autosomal recessive cases of AOS and only five *DOCK6*-related families have been reported to date. Recently, a second type of autosomal recessive AOS has been attributed to *EOGT* mutations in three consanguineous families. Here, we describe the identification of 13 *DOCK6* mutations, the majority of which are novel, across 10 unrelated individuals from a large cohort comprising 47 sporadic cases and 31 AOS pedigrees suggestive of autosomal recessive inheritance. *DOCK6* mutations were strongly associated with structural brain abnormalities, ocular anomalies, and intellectual disability, thus suggesting that *DOCK6*-linked disease represents a variant of AOS with a particularly poor prognosis.

Keywords: Adams-Oliver syndrome, *DOCK6*, autosomal recessive, brain anomalies, eye anomalies.

First described in 1945, Adams-Oliver syndrome (AOS) is characterized by the combination of terminal transverse limb defects (TTLD) and aplasia cutis congenita (ACC) typically located in the midline parietal and/or occipital region of the scalp [Adams and Oliver, 1945]. Structures underlying these defects (skull bones, meninges, sinus) may also be involved. AOS is often associated with additional congenital vascular anomalies such as cutis marmorata telangiectatica congenita (CMTC), reported in around 20% of patients, pulmonary hypertension, and lesions of presumed vascular etiology in other organs. Moreover, around 20% of patients with AOS have congenital cardiac defects including – amongst others – aortic valve anomalies, septal defects and tetralogy of Fallot [Snape et al., 2009]. The spectrum of congenital anomalies observed in AOS has led to the hypothesis that disturbed vasculogenesis may underlie this disorder [Swartz et al., 1999]. AOS is emerging as a very heterogeneous disorder, both clinically and genetically. To date, three genes have already been identified as causative for the autosomal dominant form, namely *ARHGAP31* (MIM *610911; AOS1; #100300) [Southgate et al., 2011], *RBPJ* (MIM *147183; AOS3; #614814) [Hassed et al., 2012], and *NOTCH1* (MIM *190198; AOS5; #616028) [Stittrich et al., 2014]. Two genes, *DOCK6* (MIM *614194; AOS2; #614219) [Shaheen et al., 2011] and *EOGT* (MIM *614789; AOS4; #615297) [Shaheen et al., 2013], have been reported in pedigrees with autosomal recessive transmission of AOS. Each of these genes apparently accounts for only a minor proportion of patients. It is therefore likely that further AOS genes will be identified in the future.

Homozygous or compound heterozygous *DOCK6* mutations have so far been reported in only four inbred Arab families [Shaheen et al., 2011, 2013] and in a single sporadic patient [Lehman et al., 2014], respectively. The DOCK6 protein belongs to the conserved dedicator of cytokinesis family and has a role in remodeling the actin cytoskeleton by acting as a guanine nucleotide exchange factor (GEF) for two members of the Rho GTPase family, Cdc42 and Rac1 [Miyamoto et al., 2007]. This regulation of Cdc42 and Rac1 complements

the GTPase-activating protein (GAP) activity of the gene product of *ARHGAP31* [Tcherkezian et al., 2006], mutations of which underlie some autosomal dominant cases of AOS [Southgate et al., 2011; Isrie et al., 2014], thus pointing at abnormal cytoskeleton remodeling as one of the basic pathogenic mechanisms leading to AOS.

To further understand the role of *DOCK6* in the etiology of this disorder and to establish possible phenotype correlations, we performed a comprehensive mutation screen of this gene in a large and heterogeneous patient cohort. The study cohort consisted of 88 patients from 78 unrelated families recruited by the partners of the AOS Collaborative Group. The presence of both ACC and TTLD in at least one affected family member served as minimal clinical inclusion criteria for this study, with the exception of one case that has been previously published as a variant of AOS with cognitive impairment, but without scalp defect [Brancati et al., 2008]. Additional physical abnormalities were reported in a considerable proportion of patients and included cerebral (n=19), ocular (n=13), neurodevelopmental (n=25), and cardiac anomalies (n=14). Either sporadic cases of AOS (n=47) or those with a pedigree constellation suggestive of autosomal recessive disease transmission (n=31) were included. Parental consanguinity and/or the presence of multiple affected children of clinically unaffected parents were regarded as possible indicators of autosomal recessive inheritance. Families with parent-child transmission of the phenotype suggesting autosomal dominant inheritance were excluded. *DOCK6* mutation screening was performed by PCR and conventional sequencing of all 48 coding exons and flanking intronic regions (Supp. Materials and Methods). The study was approved by the institutional review boards of the participating centers, University of Magdeburg/Erlangen, Guy's and St Thomas' Hospitals London, and University of Antwerp. Written informed consent was obtained from the patients and/or the parents. Mutations, unclassified variants and phenotype data were submitted to the Leiden Open Variation Database (http://databases.lovd.nl/).

In this cohort we detected 10 unrelated individuals with biallelic sequence changes in *DOCK6* that were classified as probable pathogenic mutations. Seven of those patients were offspring of consanguineous parents, two originated from non-consanguineous families with multiple affected children, and one was a sporadic case with no known parental consanguinity (Supp. Figure S1). The overall proportion of *DOCK6*-related AOS across our complete cohort was 13%, with a frequency of 29% (9/31) among the families suggestive of autosomal recessive inheritance and 2% (1/47) in sporadic cases with no parental consanguinity. Our findings thus underscore the importance of *DOCK6* as a gene for autosomal recessive AOS. They also suggest that a small proportion of apparently sporadic cases are in fact recessive with *DOCK6* as the underlying etiology.

The mutations observed in these 10 families included nonsense (n=1), missense (n=4), frameshift (n=4), and splice site mutations (n=3), as well as one larger intragenic deletion-insertion resulting in deletion of exons 42 to 47. The latter was identified through the failure to amplify the terminal exons by PCR and confirmed by focused MLPA and breakpoint sequencing (Supp. Figure S1, family 9). Eleven of these 13 mutations were novel and two have been previously described as causative of AOS [Shaheen et al., 2011, 2013] (Figure 1A and Supp. Table S1). Seven index patients had homozygous mutations consistent with self-stated parental consanguinity, while the remaining three had compound heterozygous changes. Of the four missense mutations observed in this cohort, three were homozygous in affected children from consanguineous families (c.3047T>C, p.Leu1016Pro; c.3154G>A, p.Glu1052Lys; c.4786C>T, p.Arg1596Trp) and one (c.788T>A, p.Val263Asp) occurred in compound heterozygosity with a splice site mutation on the second allele. All four missense variations were classified as likely causative mutations on the basis of conservation of the affected residue, as assessed by various online prediction tools (Supp. Table S2). Moreover, in the consanguineous family harboring the missense mutation p.Leu1016Pro (c.3047T>C, family 1), previous homozygosity mapping using a SNP array had been consistent with

linkage to the *DOCK6* locus in the index patient, demonstrating a 22 Mb stretch of autozygosity on chromosome 19 (data not shown). In one pedigree (family 6), segregation of compound heterozygosity for the missense mutation p.Val263Asp and a splice site mutation on the second allele (c.5939+2T>C) was confirmed in the two affected siblings (Table 1, Supp. Figure S1). Of the three splice site mutations observed in this study, one (c.4106+5G>T) is outside of the canonical splice site dinucleotide. Unfortunately, no appropriate material could be obtained to prove the splicing effect on the mRNA level. However, compound heterozygosity for this change and a frameshift mutation on the other allele was found to segregate with the phenotype in family 7 (Table 1, Supp. Figure S1). Furthermore, splice prediction tools consistently calculated that this change likely abrogated splice donor function at this site (Supp. Table S3), thus supporting the likely pathogenic role of this variation. To date, six distinct *DOCK6* mutations have been reported to underlie the AOS type 2 (Figure 1A, Supp. Table S1), with loss of function or expression of the DOCK6 protein suggested as the basic pathogenic mechanism [Shaheen et al., 2011, 2013; Lehman et al., 2014]. Taken together with previous reports, this study demonstrates that *DOCK6* mutations are distributed over the entire gene with no obvious clustering to certain domains of the encoded protein (Figure 1A). A deleterious effect on the gene product is plausible for most of these changes, as they are predicted to lead to either a truncated protein or nonsense-mediated mRNA decay. However, the precise functional consequences of the novel missense mutations presented here remain to be explored.

In addition to the pathogenic mutations described above, we also identified 16 heterozygous *DOCK6* sequence variations in our cohort, which remained as unclassified due to either uncertain clinical significance or annotation in dbSNP (build 139) as rare variants (MAF <0.01) (Supp. Table S4). These variants included predicted amino acid substitutions (n=8), synonymous alterations in the coding sequence (n=5), and intronic substitutions within 20 bp of the splice site (n=3). None of these variations were unambiguously classified as

disease-causing by prediction tools. Thirteen unrelated sporadic cases harbored a single heterozygous unclassified *DOCK6* variant, while two patients were found to have two or more variants. Of these, one case had inherited both variants (c.885C>T, p.(=) and c.2104G>A, p.Gly702Ser) from the mother on the same allele (data not shown). Another patient was found to harbor three unclassified variants (c.885C>T, p.(=); c.1289G>A, p.Arg430His; c.1833-19C>G), the segregation of which could not be studied. Notably, this patient was previously reported in the literature as a variant subtype of AOS associated with cerebral anomalies, seizures and severe MR, but without ACC of the scalp [Brancati et al., 2008]. While most of these variations are more likely to be non-pathogenic (Supp. Table S5), we cannot fully exclude any contribution to the observed phenotype. Our mutation screening strategy did not assess mutations of the promoter and intronic changes. We also did not systematically screen for larger genomic deletions/duplications. Therefore, it remains possible that additional pathogenic variants may have been missed in this cohort and that the given figure of the contribution of *DOCK6*-related disease is somewhat underestimated. However, for the *DOCK6* mutation-negative patients originating from consanguineous families we can state that five had a previous SNP array analysis showing no suggestive stretch of homozygosity at the *DOCK6* locus (data not shown). In two out of four further subjects who had no previous homozygosity mapping, *DOCK6* sequencing revealed at least one heterozygous SNP, whilst for two cases, sequencing results were uninformative in excluding homozygosity at the *DOCK6* gene locus. Thus, at least for our consanguineous families we can conclude that genes other than *DOCK6* are very likely involved in the pathogenesis of AOS. Mutations of the *EOGT* gene may account for part of our *DOCK6*-negative AOS cases [Shaheen et al., 2013]; however mutation screening of this gene was not within the scope of this study. It also remains to be seen whether further recessive AOS genes will be identified in due course. Moreover, considering the inclusion criteria for this study, it is possible that a proportion of our cohort may in fact represent dominant *de novo* mutations or, in the case of

affected siblings with asymptomatic parents, autosomal dominant inheritance with incomplete penetrance.

The main clinical findings of the *DOCK6*-positive individuals from our cohort are summarized in Table 1. Detailed clinical data could be obtained from 10 patients originating from eight families. The patients' ages ranged between one week and 20 years (median 4.3 years). All except one affected individual from these families had ACC of the scalp and TTLD of variable expression; patient 7.2 presented only with mild hypoplasia of toenails along with a congenital heart defect, impaired vision and mild cognitive impairment, whereas his sister presented with classic AOS features including ACC and TTLD. Across our *DOCK6*-positive cohort, the limb defects ranged from minimal hypoplasia of terminal phalanges to severe transverse reduction defects (Figure 1B). Notably, aside from ACC typically located on the scalp vertex, four patients had additional areas of ACC on the abdomen. Further associated anomalies, primarily related to the nervous system, were present in all individuals carrying homozygous or compound-heterozygous *DOCK6* mutations. Specifically, all patients from whom sufficient data could be obtained were reported with developmental delay or mental retardation, ranging from mild to severe (Table 1). A broad range of additional neurological abnormalities were reported in most cases, including cerebral palsy, spasticity, contractures, and epilepsy. Only one patient aged ≥4 years had achieved the ability to walk without support. Behavioral abnormalities including autistic behavior or temper tantrums were reported in two patients. Brain MRI or CT had been performed for seven patients and was abnormal in all cases. The most frequent changes observed on brain imaging included ventriculomegaly, periventricular leukomalacia/calcifications, and hypoplasia/atrophy of the corpus callosum (Table 1). Images from five affected individuals are exemplarily shown in Figure 1C. Patient 4.1 underwent cerebral ultrasonography at 3 months of age which also showed ventriculomegaly. A further patient (6.2) was previously reported with ventricular dilatation, partial agenesis of the corpus callosum, and periventricular leukomalacia on

autopsy [Orstavik et al., 1995]. Where available, measurements of head circumference were in the microcephalic range for all eight patients. Ocular anomalies including microphthalmia, retinal detachment, and visual impairment were reported in all patients for whom clinical information was obtainable. In contrast, cardiac anomalies were observed in only three cases.

Taken together, the most striking phenotypic attribute of *DOCK6*-related AOS in the presented cohort is the strong association with important neurodevelopmental and ocular anomalies. The pattern of neurological impairment and most of the reported morphological changes (microcephaly, ventricular dilatation, periventricular calcifications, cortical changes) are suggestive of a disruptive vascular pathogenesis rather than a primary maldevelopment of the brain. Lesions classified as calcifications according to density analysis, may represent primary calcifications but can in fact also have resulted from previous microbleeds. Likewise, the main ocular anomalies observed in our *DOCK6*-positive patients, namely microphthalmia and retinal detachment, are compatible with a disruptive vasculogenesis. The high prevalence of brain and eye abnormalities as well as the pattern of cerebral and ocular involvement is in line with previous case reports (Table 1). However the data on the previously reported patients do not provide specific detail to definitely state that brain involvement is a constant feature in AOS type 2. While *DOCK6* mutations are generally a rare cause of AOS, in our cohort they accounted for 8/25 (32%) cases presenting with major neurodevelopmental defects and for 9/19 (47%) cases with documented brain abnormalities. Taken together, these data suggest that *DOCK6* mutations are particularly responsible for a variant of AOS characterized by ACC, TTLD plus cerebral and ocular abnormalities. The existence of such a variant was previously postulated nearly 20 years ago [Orstavik et al., 1995] and our study now confirms that *DOCK6* is indeed the gene responsible for the disease in that family (family 6). The strong association of *DOCK6* mutations with anomalies of the brain and eye implies that deleterious effects on angiogenesis caused by DOCK6 deficiency also affect development of these particular structures. In their review, Snape et al. concluded that

abnormal brain and ocular findings are more common in autosomal recessive AOS [Snape et al., 2009]. It is becoming clear that the individuals with *DOCK6* mutations account for a substantial part for this observation. By contrast, among five patients with *EOGT* mutations, only one patient was reported to have brain anomalies and no abnormal ocular findings were reported in any subject [Shaheen et al., 2013]. Nonetheless, across our complete cohort, approximately two-thirds of the AOS patients with major neurodevelopmental disorders and about half of the cases with structural brain anomalies could not be explained by *DOCK6* mutations, thus suggesting that the association with a neurological phenotype is not specific to AOS type 2.

In summary, by presenting 10 novel families with *DOCK6* mutations, we substantially expand the clinical and mutational spectrum of AOS type 2. Our findings provide independent corroboration that mutations in *DOCK6* are responsible for nearly one third of autosomal recessively inherited AOS and that this genetic entity also accounts for a minority of sporadic cases. AOS type 2 is particularly if not consistently associated with cerebral and ocular anomalies in addition to ACC and TTLD. In patients with such a constellation of symptoms *DOCK6* should therefore be the primary candidate gene for molecular investigation.

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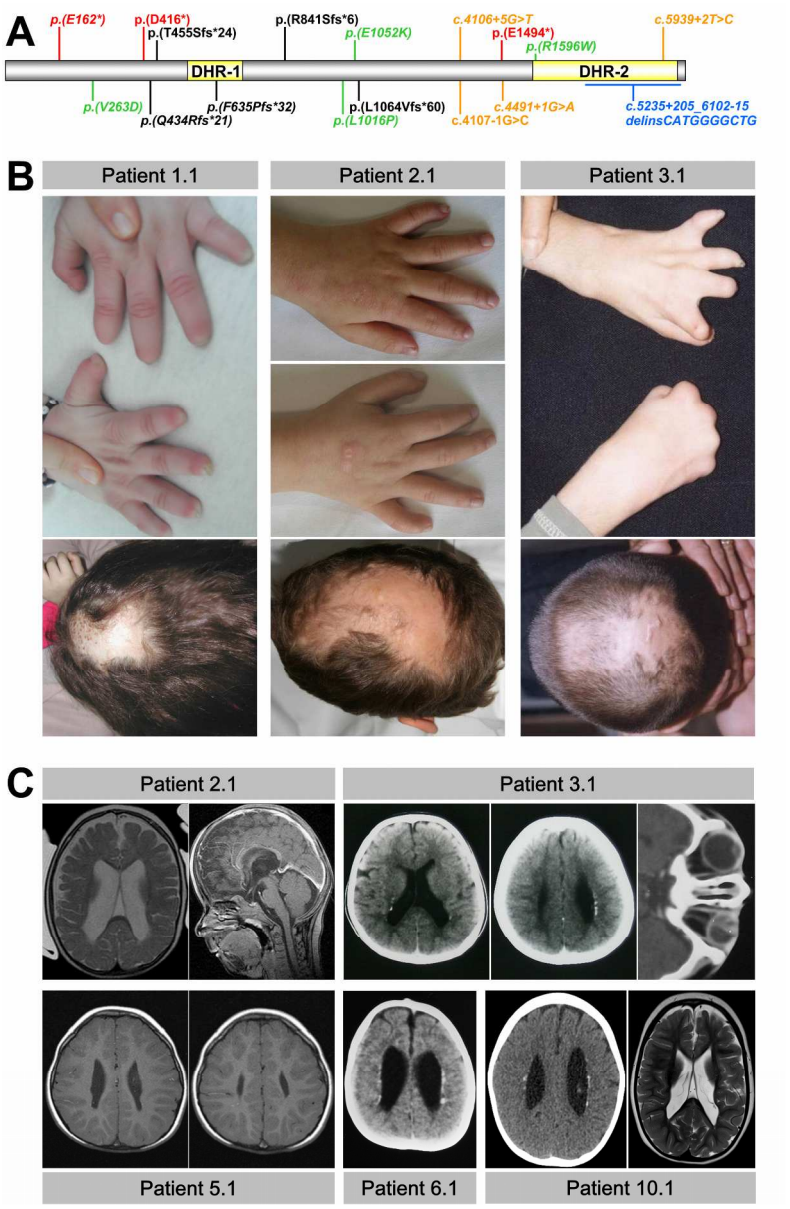
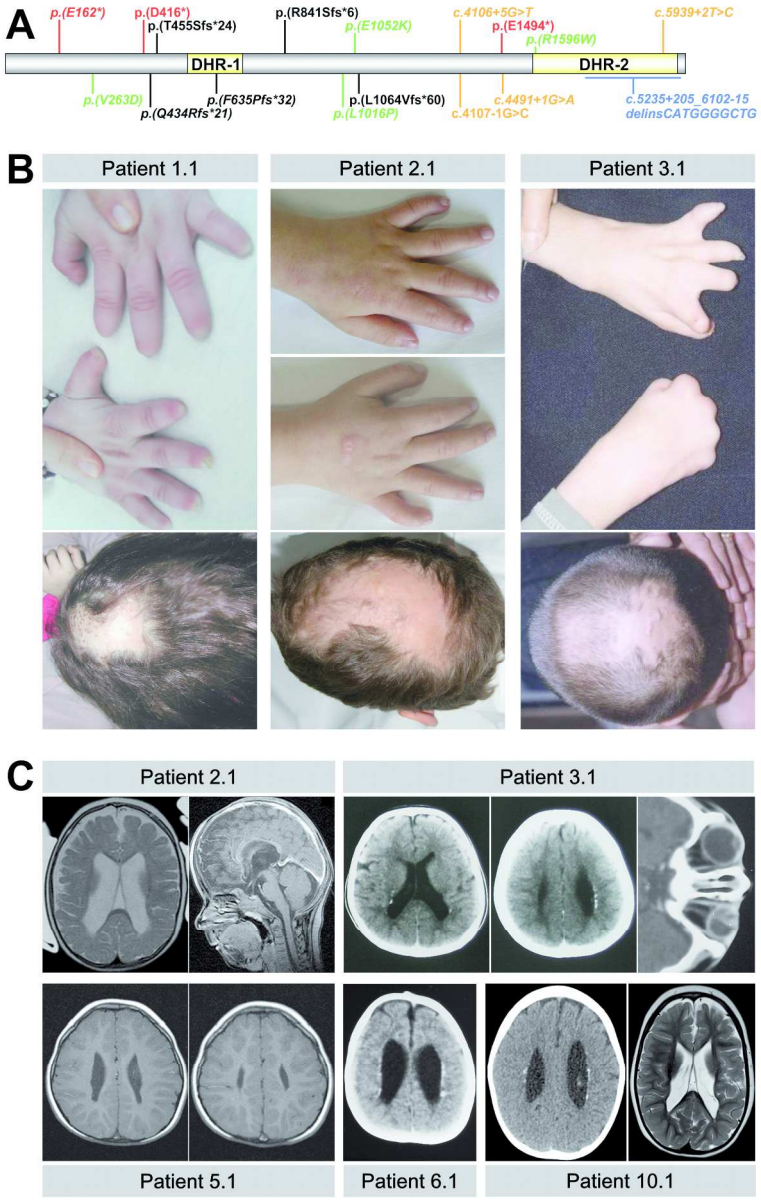
Figure Legend

Figure 1: (**A**) DOCK6 protein with known functional domains and distribution of mutations. The protein contains two DOCK homology regions, DHR-1 and DHR-2. DHR-1 spans about 200 amino acids at the N-terminal end of the protein, whereas DHR-2 is located towards the C-terminus and has an approximate length of 500 amino acids [Cote and Vuori, 2002]. All currently known mutations are displayed according to their location in the DOCK6 protein. Red represents nonsense mutations (n=3), black indicates frameshift mutations (n=5), missense mutations are shown in green (n=4), splice site mutations are colored in orange (n=4) and the blue line represents one large deletion insertion at the C-terminal end of the DOCK6 protein spanning exons 42 to 47. Novel mutations reported in this paper are written in italics. (**B**) Clinical photographs of three *DOCK6*-positive individuals with AOS from this cohort showing areas of alopecia on the vertex resulting from aplasia cutis congenita and terminal defects of the digits of varying severity. (**C**) Brain imaging of AOS patients with *DOCK6* mutations. Cranial MRI of **patient 2.1** at age 1 year: T2-weighted axial section showing enlarged lateral ventricles and cerebral atrophy particularly affecting the frontal lobe, and contrast enhanced T1-weighted median sagittal section illustrating thin corpus callosum and enlarged basal subarachnoid spaces. CT scan of **patient 3.1** at age 6 years: Axial sections showing ventriculomegaly and periventricular calcifications, and orbital section showing right microphthalmia with interocular hyperdensities representing retinal detachment and cystic malformation of the anterior chamber. T1-weighted MRI of **patient 5.1** at age 3 years: Axial sections showing irregularly shaped and slightly dilated lateral ventricles. Axial CT scan of **patient 6.1** in neonatal period showing ventricular dilatation and multiple periventricular calcifications. Brain imaging of **patient 10.1**: CT scan at age 2 years showing periventricular calcifications, and T2-weighted MRI axial section age 3 years showing irregularly shaped, slightly enlarged ventricles and mild atrophy of the brain. MRI, magnetic resonance imaging, CT, computed tomography.



(A) DOCK6 protein with known functional domains and distribution of mutations.
(B) Clinical photographs of three *DOCK6*-positive individuals with AOS from this cohort showing areas of alopecia on the vertex resulting from aplasia cutis congenita and terminal defects of the digits of varying severity.
(C) Brain imaging of AOS patients with *DOCK6* mutations.
[RGB color]
195x299mm (300 x 300 DPI)



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[CMYK color]
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Table 1: Mutation and phenotype data of *DOCK6*-positive individuals from this cohort (families 1-10) compared to previously published cases (families 11-15).

Family	Patient	Mutations	Gender	Age	Parental consanguinity	Intra uterine growth restriction	Scalp defect	TTLD [hands/feet]	Congenital heart defect	Brain anomalies	Microcephaly	Ocular anomalies	Cognitive impairment	Neurology	Additional features	Reference
1	1.1	[p.L1016P] + [p.L1016P]	F	5y	+	na	+	+/+	na	na	+	MO, RD, VO, ACA	DD	SE	high palate	-
2	2.1	[p.T455Sfs*24] + [c.4491+1G>A]	M	10y	-	-	+	+/+	na	VD/BA, CCH	+	NS	sev ID	SE, CP	CMTC, single umbilical artery, cryptorchidism	-
3	3.1	[p.Q434Rfs*21] + [p.Q434Rfs*21]	M	20y	+	+	+	+/+	-	VD/BA, PVL	+	MO, RD, ACA	sev ID	SE, CP	CMTC, abdominal skin defect	-
4	4.1	[p.R1596W] + [p.R1596W]	F	3m	+	-	+	+/+	PDA	VD/BA	+	MO	na	-	knee dislocation	-
5	5.1	[p.E1052K] + [p.E1052K]	M	9y	+	+	+	+/+	-	VD/BA, CCH, PVL	+	MO, RD	mod ID	SE	cryptorchidism	1
6	6.1	[p.V263D] + [c.5939+2T>C]	F	na	-	-	+	+/+	VSD	VD/BA, PVL	+	MO, RD, VO	sev ID	SE, CP	abdominal skin defects, absence of right patella	2
	6.2	[p.V263D] + [c.5939+2T>C][a]	M	1w[†]	-	+	+	+/+	na	VD/BA, CCH	na	RD	na	na	abdominal skin defect, patella fixed to skin	2
7	7.1	[p.F635Pfs*32] + [c.4106+5G>T]	F	7y	-	-	+	+/+	-	NS	+	NS	sev ID	SE	abdominal skin defect	-
	7.2	[p.F635Pfs*32] + [c.4106+5G>T]	M	8y	-	+	-	-/+	TAPVD	na	na	NS	mild ID	-	hypothyroidism	-
8	8.1	[p.E162*] + [p.E162*]	F	na	+	na	+	+/+	na	na	na	na	na	na		-
9	9.1	[c.5235+205_6102-15delins10] + [c.5235+205_6102-15delins10]	F	7y	+	-	+	+/+	na	PVL	na	na	na	na		-
10	10.1	[p.R841Sfs*6] + [p.R841Sfs*6]	F	na	+	-	+	+/+	na	VD/BA, CCH, PVL	+	na	na	SE		-
11	11.1	[p.T455Sfs*24] + [p.T455Sfs*24]	F	11m	+	na	+	+/+	-	VD/BA, PVL	+	OA	sev ID	SE, CP		3
12	12.1	[p.D416*] + [p.D416*]	F	3.5y	+	na	+	+/+	-	na	+	-	DD	na		3
13	13.1	[p.R841Sfs*6] + [p.R841Sfs*6]	M	1y	+	na	+	+/+	AVD	VD/BA, PVL	na	-	na	na	abdominal skin defect	4
	13.2	[p.R841Sfs*6] + [p.R841Sfs*6]	F	na	+	na	+	+/+	na	VD/BA, PVL	na	na	na	SE	gastroschisis	4
14	14.1	[c.4107-1G>C] + [c.4107-1G>C]	F	2y	+	na	+	+/+	na	PVL, PA	na	OA	na	SE		4
15	15.1	[p.L1064Vfs*60] + [p.E1494*]	F	2y	-	+	+	+/+	TOF, PLSVC	PVL, PE	+	RD	sev ID	SE	placental vasculopathy, neonatal thrombocytopenia, small bowel infarction	5

[a]Genotype was not directly confirmed as patient is deceased but is assumed to be the same as in affected sibling.

F, female; M, male; y, year(s); m, month(s); w, weeks(s); †, deceased; na, no data available; +, present; -, not present; TTLD, terminal transverse limb defects; PDA, patent ductus arteriosus; VSD, ventricular septal defect; TAPVD, total anomalous pulmonary venous connection; AVD, aortic valve dysplasia; TOF, tetralogy of Fallot; PLSVC, persistent left superior vena cava; VD/BA, ventricular dilatation / brain atrophy; CCH, corpus callosum hypoplasia/atrophy; PVL, periventricular lesions (calcification, gliosis); NS, abnormality present, not further specified; PA, pachygyria; PE, porencephaly; MO, microphthalmia; RD, retinal detachment; VO, vitreous opacities/membranes; ACA, anterior chamber abnormality; OA, optic atrophy; DD, developmental delay; ID, intellectual disability; sev, severe; mod, moderate; SE, seizures / epilepsy; CP, cerebral palsy / spasticity; CMTC, cutis marmorata telangiectatica congenita. References: (1) Prothero et *al*. (2007); (2) Orstavik et *al*. (1995); (3) Shaheen et *al*. (2011); (4) Shaheen et *al*. (2013); (5) Lehman et *al*. (2014).

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DOCK6 Mutations are Responsible for a Distinct Autosomal Recessive Variant of Adams-Oliver Syndrome Associated with Brain and Eye Anomalies

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Supp. Material and Methods

We designed oligonucleotide primers for each of the 48 exons of *DOCK6* using the Primer3 software version 4.0.0 (http://primer3.ut.ee/) [Untergrasser et al., 2012]. Primer sequences and PCR conditions are available upon request. Sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI 3500xl automated capillary sequencer (Applied Biosystems, Cheshire, UK). Obtained sequences were compared with the reference sequence (NM_020812.3) using the Sequence Pilot software v4.0.1 (JSI Medical Systems GmbH, Kippenheim, Germany). Pathogenicity of all observed sequence variants was assessed using various online prediction tools. For splice site prediction we utilized the following bioinformatic tools:

- BDGP (Berkeley Drosophila Genome Project)
  last updated 28 July 2014, Human or other, minimum scores for splice sites: 0.1
  http://www.fruitfly.org/seq_tools/splice.html [Reese et al., 1997]
- NetGene2
  Version 2.4, Human
  http://www.cbs.dtu.dk/services/NetGene2/ [Brunak et al., 1991]

Missense mutations were rated using the following:

- PolyPhen-2 (Polymorphism Phenotyping v2)
  Version 2.2.2, NP_065863.2
  http://genetics.bwh.harvard.edu/pph2/ [Adzhubei et al., 2010]
- SIFT Human Protein (Sorting Intolerant From Tolerant)
  page last modified: August 2011, Ensembl 63, ENSP00000294618
  http://sift.jcvi.org/www/SIFT_enst_submit.html [Ng and Henikoff, 2003]

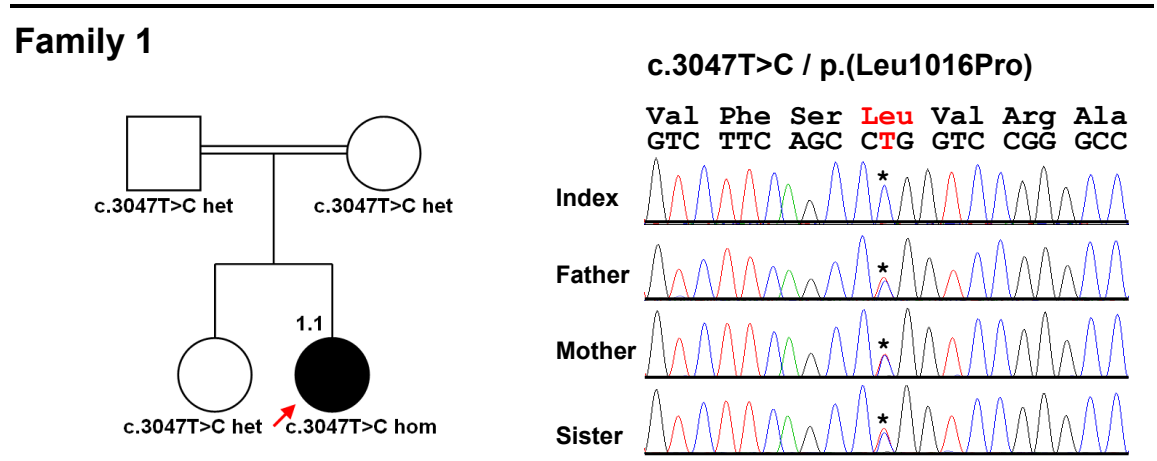
- MutPred (Mutation Prediction)
  last modified 02 Feb 2014, NP_065863.2
  http://mutpred.mutdb.org [Li et al., 2009]
- GERP (Genomic Evolutionary Rate Profiling)
  hg19,
  http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html [Cooper et al., 2005]

All variants were checked regarding their appearance/frequency in EVS (Exome Variant Server, Gene ID: 57572, GRCh37, http://evs.gs.washington.edu/EVS/, [Exome Variant Server, 2015]) and TGP (1000 Genomes Project, http://www.1000genomes.org/home, [1000 Genomes Project Consortium et al., 2010]). Protein conservation across species was checked by Standard Protein BLAST (Basic Local Alignment Search Tool, Database: Reference proteins (refseq_protein), Algorithm: blastp (protein-protein BLAST), http://blast.ncbi.nlm.nih.gov/Blast.cgi, [Altschul et al., 1990]). Segregation of the variants across family members was checked if appropriate material was available. Designation of mutations follows the guidelines of the Human Genome Variation Society (last modified March 2014; http://www.hgvs.org/mutnomen/) [den Dunnen and Antonarakis, 2000] and was verified by Mutalyzer (Version 2.0 beta-24; https://mutalyzer.nl/) [Wildeman et al., 2008].

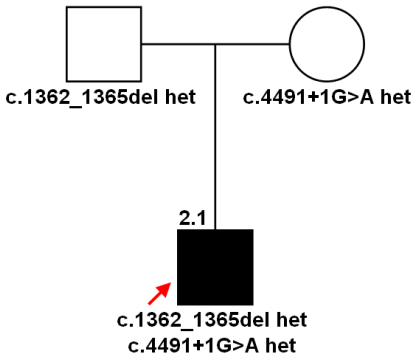
A focused MLPA assay including probes for *DOCK6* exons 40 through 48 was developed (probe sequences available upon request) for copy number determination of the terminal *DOCK6* exons in a family where PCR amplification of exons 42 to 48 failed in the index patient.

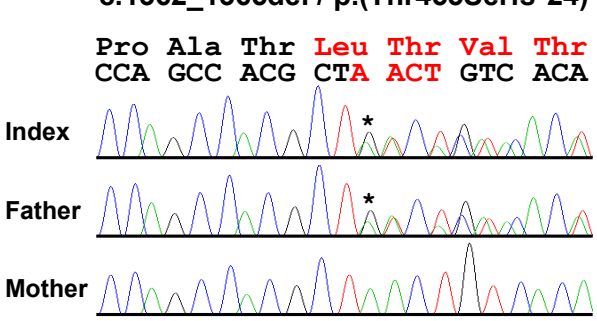
We have established a collection of all mutations and selected unclassified variants in the *DOCK6* gene (http://databases.lovd.nl/shared/variants/DOCK6) at the Leiden Open Variation Database (LOVD, version 3.0) [Fokkema et al., 2011], as well as all available phenotype data of patients that were designated as AOS2 both clinically and genotypically (http://databases.lovd.nl/shared/individuals/DOCK6). To date, the database contains 17 different *DOCK6* mutations and phenotype data from 18 individuals.

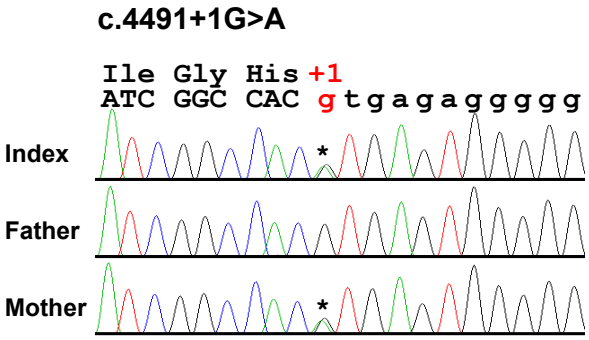
Supp. Figure S1: Pedigrees and electropherograms of *DOCK6*–positive AOS patients and their families.



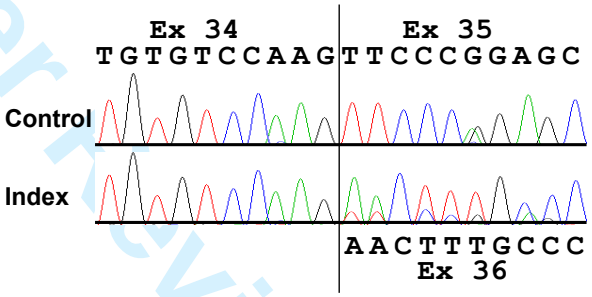
Family 2







RNA analysis



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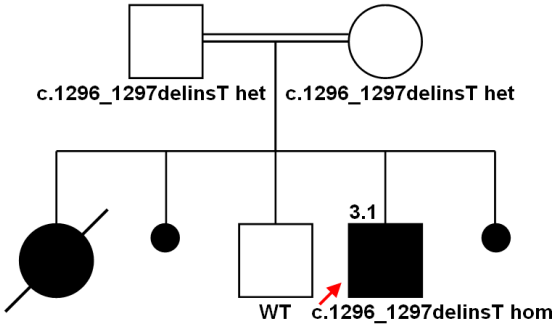
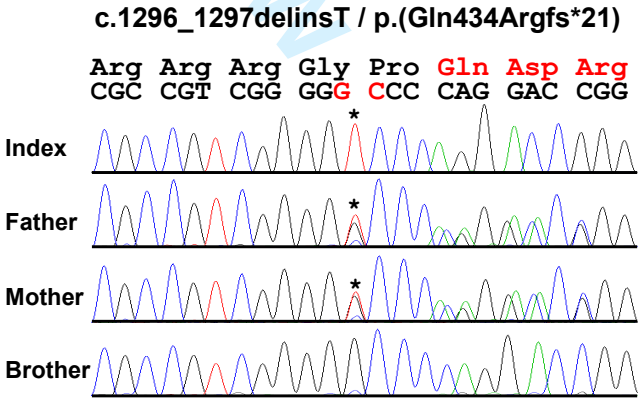
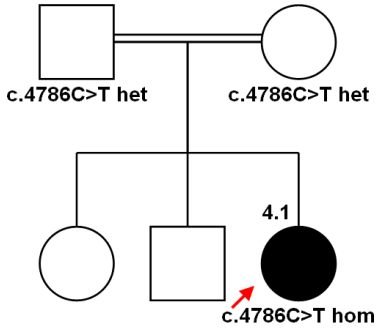
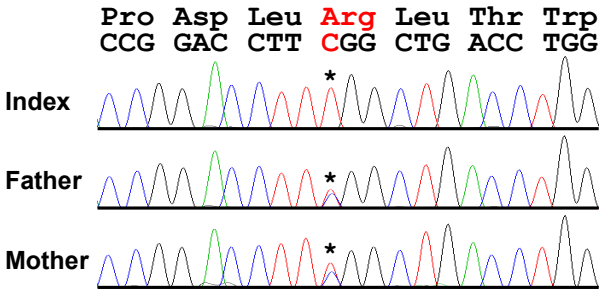


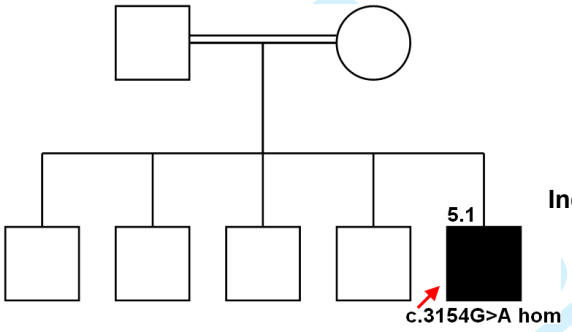


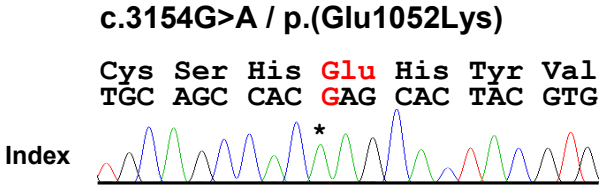
Family 4



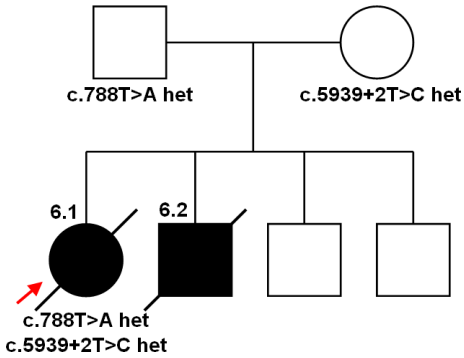


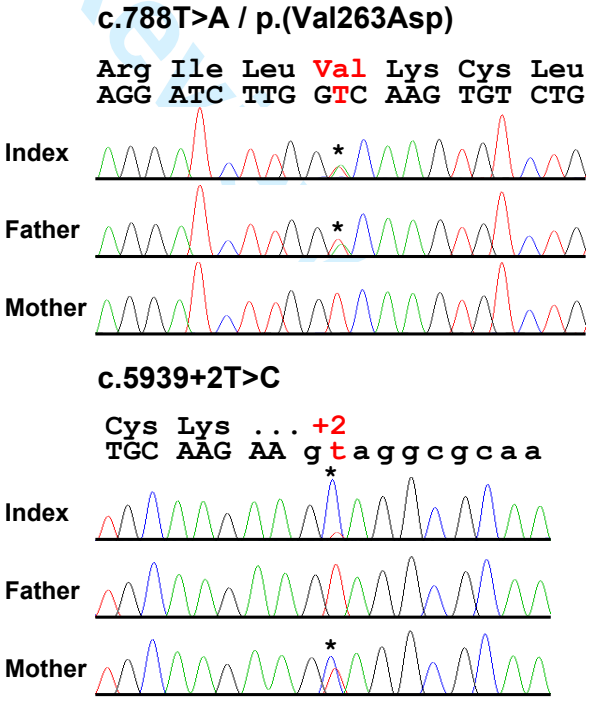
Family 5





Family 6





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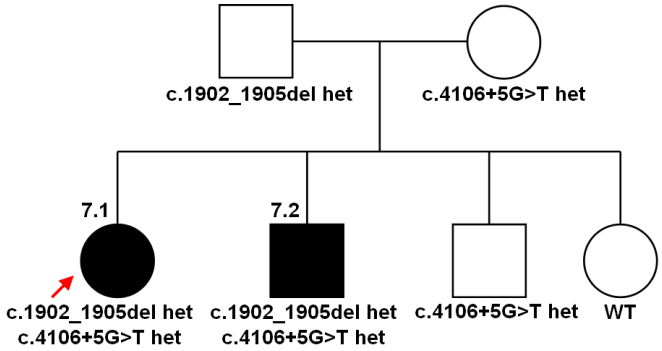
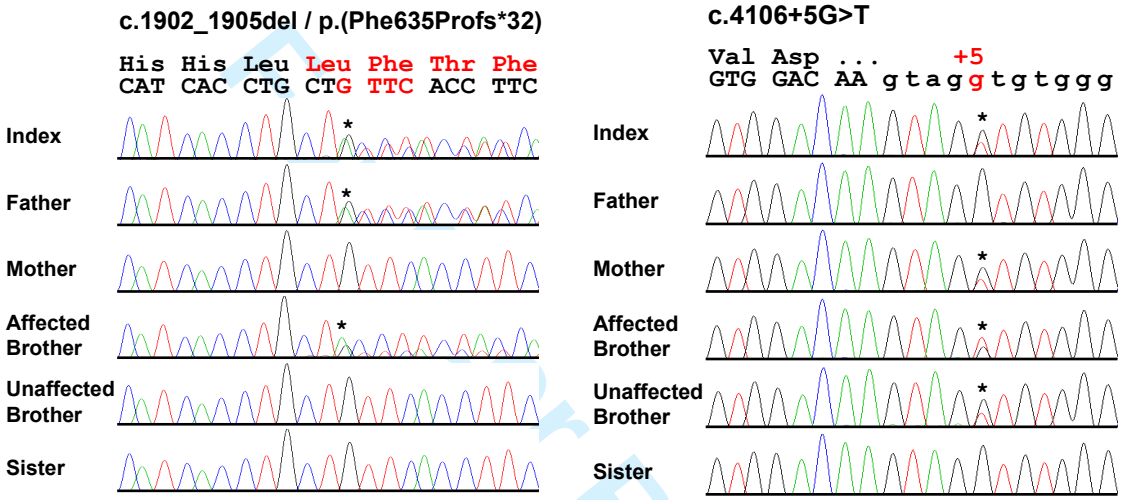
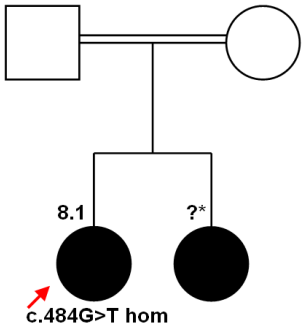
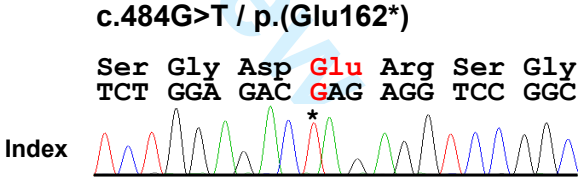
Family 7

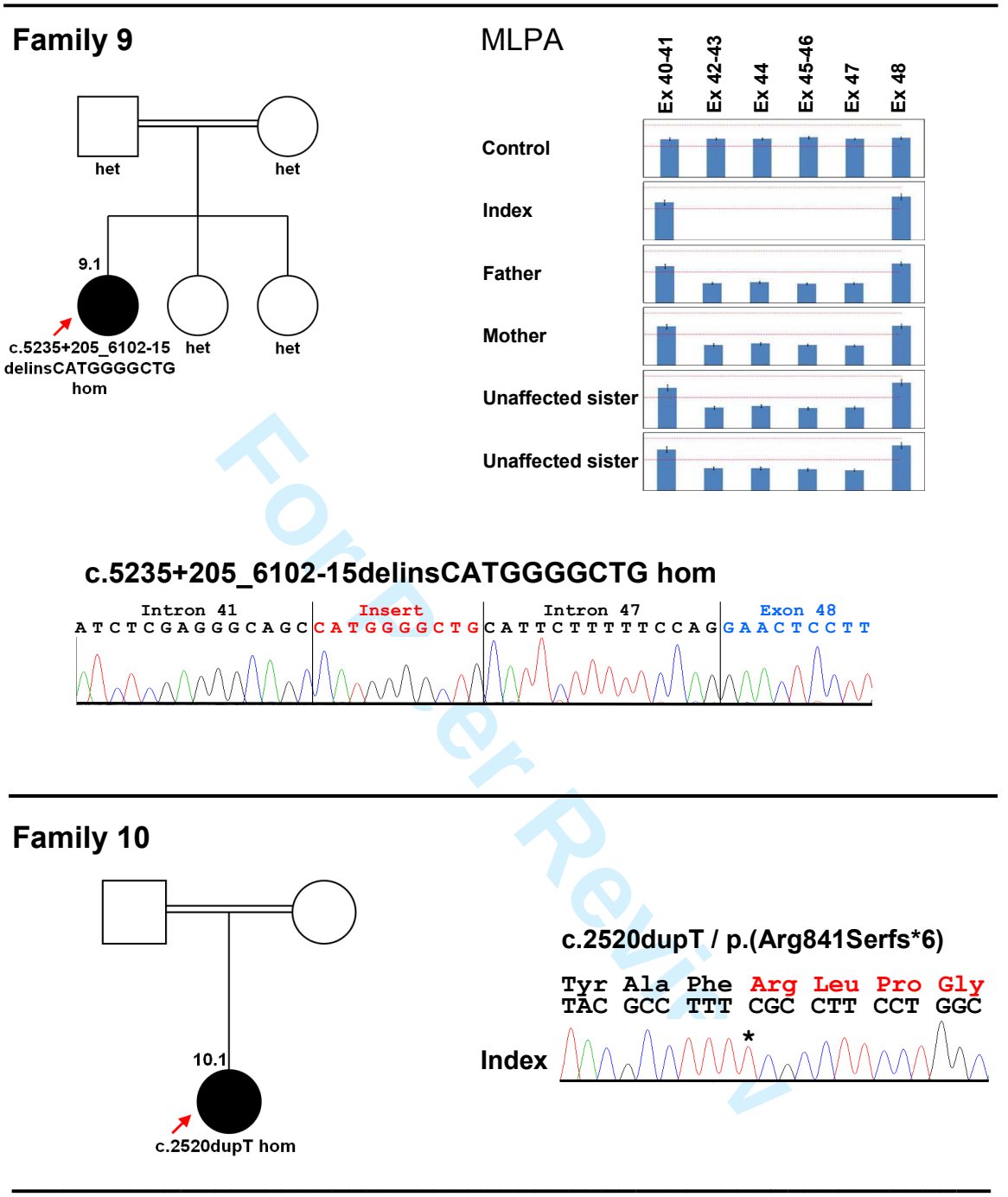




Family 8







Pedigrees representing *DOCK6* mutation positive families. Affected individuals are indicated by filled symbols. All available genotype data is added and sequence electropherograms are shown. Family 2: RNA analysis is additionally displayed. Family 6: in the index patient the wild type allele is drastically under-represented regarding the heterozygous c.5939+2T>C splice site mutation. We assume this to be caused by a technical artefact due to very poor DNA quality. Family 9: MLPA results (multiplex ligation-dependent probe amplification) are shown.

hom, homozygous; *het*, heterozygous; *WT*, wild type; *?** patient not listed in table 1 due to lack of clinical data and not genetically analyzed due to lack of adequate material.

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Supp. Table S1: Mutations in the *DOCK6* gene causing Adams-Oliver syndrome.

Location	Nucleotide Alteration	Predicted effect[a]	Family ID	EVS	MAF (TGP)	Reference(s)
Exon 05	c.484G>T	p.(Glu162*)	8	-	-	this paper
Exon 07	c.788T>A	p.(Val263Asp)	6	-	-	this paper
Exon 11	c.1245dupT	p.(Asp416*)	12	-	-	Shaheen et al. [2011]
Exon 12	c.1296_1297delinsT	p.(Gln434Argfs*21)	3	-	-	this paper
Exon 12	c.1362_1365del	p.(Thr455Serfs*24)	2, 11	0.26 %	-	this paper + Shaheen et al. [2011]
Exon 17	c.1902_1905del	p.(Phe635Profs*32)	7	-	-	this paper
Exon 21	c.2520dupT	p.(Arg841Serfs*6)	10, 13	-	-	this paper + Shaheen et al. [2013]
Exon 25	c.3047T>C	p.(Leu1016Pro)	1	-	-	this paper
Exon 26	c.3154G>A	p.(Glu1052Lys)	5	-	-	this paper
Exon 26	c.3190_3191del	p.(Leu1064Valfs*60)	15	0.02 %	-	Lehman et al. [2014]
Intron 32	c.4106+5G>T	r.spl.? p.?	7	-	-	this paper
Intron 32	c.4107-1G>C	*p.Thr1370Metfs*19*	14	-	-	Shaheen et al. [2013]
Exon 35	c.4480G>T	p.(Glu1494*)	15	-	-	Lehman et al. [2014]
Intron 35	c.4491+1G>A	r.spl.? p.?	2	-	-	this paper
Exon 38	c.4786C>T	p.(Arg1596Trp)	4	-	-	this paper
Intron 41 – Intron 47	c.5235+205_6102-15 delinsCATGGGGCTG	p.?[b]	9	-	-	this paper
Intron 46	c.5939+2T>C[c]	r.spl.? p.?	6	0.02 %	-	this paper

[a]Italic letters indicate that the effect of splicing mutations was demonstrated on the mRNA level.
[b]MLPA analysis revealed deletion of exons 42 to 47.
[c]This alteration is also listed in dbSNP (rs201387914) with unknown pathogenicity and frequency. Online tools predict destruction of the donor splice site.
Mutation nomenclature refers to GenBank reference sequence NM_020812.3. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
EVS (Exome Variant Server): frequency of alterations was calculated according to the total allele count; MAF (minor allele frequency); TGP (1000 Genomes Project): no entries for these alterations.

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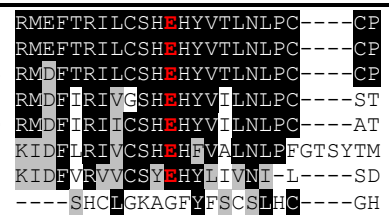
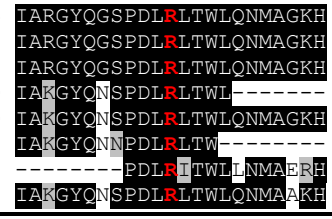
Supp. Table S2: Prediction of pathogenicity and conservation of *DOCK6* missense mutations.

Nucleotide alteration	Predicted effect	PolyPhen-2 HumVar [sensitivity/specificity]	SIFT [score/median information content]	MutPred	GERP	BLAST Alignment
c.788T>A	p.V263D	probably damaging (0.998) 0.18 / 0.98	damaging 0.00 / 2.75	0.735	5.05	
c.3047T>C	p.L1016P	probably damaging (0.977) 0.58 / 0.94	damaging 0.00 / 2.71	0.756	4.84	
c.3154G>A	p.E1052K	probably damaging (0.999) 0.09 / 0.99	damaging 0.00 / 2.71	0.492	4.81	
c.4786C>T	p.R1596W	probably damaging (0.999) 0.09 / 0.99	damaging 0.00 / 2.71	0.715	4.99	

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Various online prediction tools were used to evaluate mutation effects. **PolyPhen-2**: score >0.909, probably damaging; score 0.447 – 0.908, possibly damaging; score ≤0.446, benign; sensitivity: True Positive Rate, the chance that the mutation is classified as damaging when it is indeed damaging; specificity: 1 – False Positive Rate, the chance that the benign mutation is correctly classified as benign [Adzhubei et al., 2013]. **SIFT**: score = normalized probability that the amino acid change is tolerated; ≤0.05, damaging; >0.05, tolerated; median information content: maximum 4.32, indicates complete conservation at this position; minimum 0.00, indicates a position where all 20 amino acids are tolerated; ideally between 2.75 and 3.5 [Ng and Henikoff, 2003]. **MutPred**: general score; ranges between 1.000 (deleterious mutation) and 0.000 (benign). **GERP**: ranges from 6.17 (highly conserved amino acid residue) to -12.3 (not conserved). **BLAST Alignment**: multiple protein alignment of human DOCK6 and its orthologues; numbers indicate position of affected amino acid residue. Black shading indicates identical amino acid residues; grey shading indicates similar residues (according to BLOSUM62 matrix). Human: *Homo sapiens*, ENSP00000294618.6. Mmulatta: *Macaca mulatta*, ENSMMUP00000012229.2. Mmusculus: *Mus musculus*, ENSMUSP00000034728.7. Trubripes: *Takifugu rubripes*, ENSTRUP00000027209.1. Drerio: *Danio rerio*, ENSDARP00000077379.4. Dmelanogaster: *Drosophila melanogaster*, FBpp0077762.3. Celegans: *Caenorhabditis elegans*, F46H5.4.1. Xtropicalis: *Xenopus tropicalis*, ENSXETP00000036553.2.

Supp. Table S3: Splice site prediction c.4106+5G>T.

		Donor splice site predictions	
	+5	BDGP	NetGene2
Wild type	CGCGTGGACAAgtaggtgtgggcaggagggt	0.77	0.881
Mutant	CGCGTGGACAAgtagttgtgggcaggagggt	0.15	0.341

This donor splice site alteration at position +5 in intron 32 was detected in compound heterozygosity with a frameshift mutation in family 7. The online tools BDGP (Berkeley Drosophila Genome Project) and NetGene2 predict this nucleotide exchange to impair the regular splice donor site at the border between exon 32 and intron 32. The indicated probability scores refer to the authentic splice site.

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Supp. Table S4: Unclassified variants in the *DOCK6* gene.

Location	Nucleotide Alteration[a]	Predicted effect	dbSNP (139)	EVS	MAF (TGP)
Exon 02	c.100C>G	p.(His34Asp)	rs201065561	-	0.0004/2
Exon 09	c.885C>T	p.(=)	rs146599144	0.46 %	0.0048/24
Exon 12	c.1289G>A	p.(Arg430His)	rs143655255	0.30 %	0.0028/14
Exon 12	c.1358C>T	p.(Thr453Met)	-	-	-
Exon 13	c.1445C>T	p.(Pro482Leu)	-	-	-
Intron 16	c.1833-19C>G	r.spl.? p.?	rs188183013	0.22 %	0.0028/14
Exon 19	c.2104G>A	p.(Gly702Ser)	rs199838752	0.08 %	0.0020/10
Exon 23	c.2767G>A	p.(Val923Ile)	rs143194982	0.02 %	0.0002/1
Exon 30	c.3873C>T	p.(=)	rs200843111	0.03 %	-
Exon 31	c.3913C>T	p.(Arg1305Cys)	rs112911897	0.70 %	0.0050/25
Exon 37	c.4732C>T	p.(Leu1578Phe)	-	-	-
Exon 38	c.4899G>A	p.(=)	rs72985308	0.20 %	0.0010/5
Exon 41	c.5229C>A	p.(=)	rs56243833	0.21 %	0.0026/13
Exon 44	c.5640C>T	p.(=)	rs200959822	0.11 %	0.0004/2
Intron 44	c.5688+9G>C	r.spl.? p.?	-	0.09 %	0.0002/1
Intron 45	c.5833-16C>G	r.spl.? p.?	rs199764395	0.05 %	0.0002/1

Only variants within 20 bp of the exons and MAF ≤ 0.01 were included. All variants show no alteration of splice site prediction in BDGP and NetGene2. None of these variants appeared as homozygous in TGP (1000 Genomes Project). The numbering for the nucleotide changes are based on cDNA sequence in accordance with the GenBank entry NM_020812.3 (GRCh37). [a]For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence.
EVS: Exome Variant Server, frequency of alterations was calculated according to the total allele count; MAF: minor allele frequency [frequency of alternative nucleotide / total allele count of alternative nucleotide].

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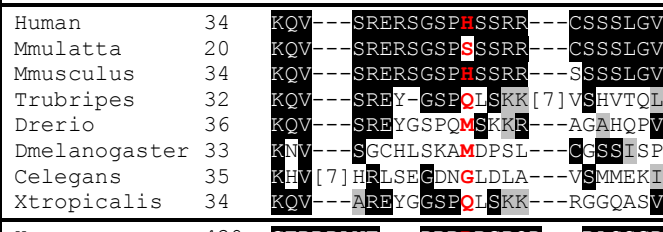
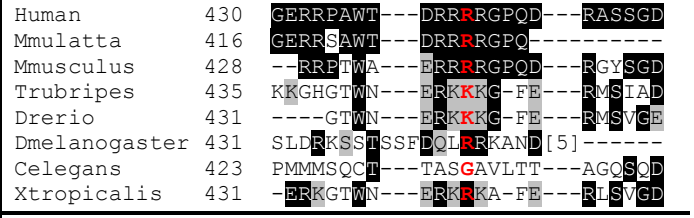
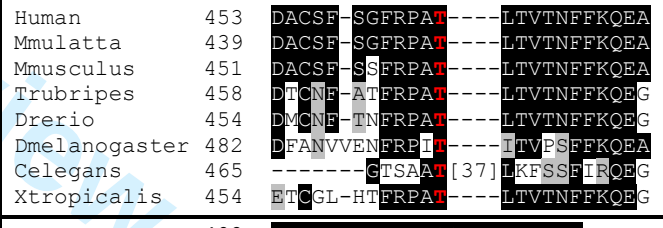
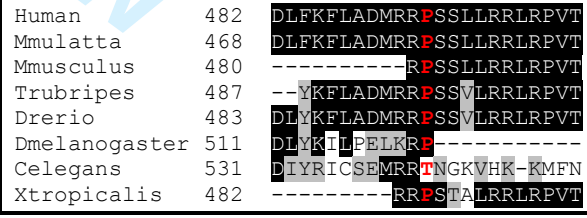
Supp. Table S5: Prediction of pathogenicity and conservation of *DOCK6* unclassified missense variants.

Nucleotide alteration	Predicted effect	PolyPhen-2 HumVar [sensitivity/specificity]	SIFT [Score/Median information content]	MutPred	GERP	BLAST Alignment
c.100C>G	p.H34D	benign (0.017) 0.95 / 0.54	tolerated 0.34 / 2.77	0.155	1.86	
c.1289G>A	p.R430H	possibly damaging (0.512) 0.82 / 0.81	tolerated 0.12 / 2.78	0.334	3.11	
c.1358C>T	p.T453M	probably damaging (0.974) 0.59 / 0.93	damaging 0.00 / 2.78	0.303	4.15	
c.1445C>T	p.P482L	probably damaging (0.991) 0.50 / 0.95	damaging 0.00 / 2.76	0.416	4.42	

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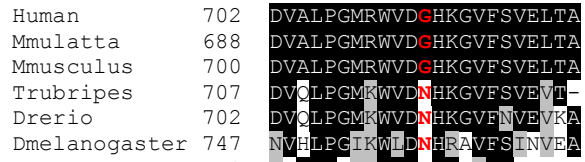
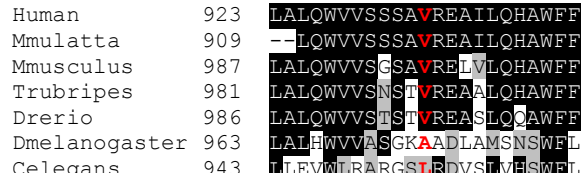
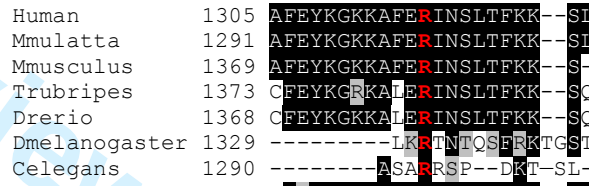
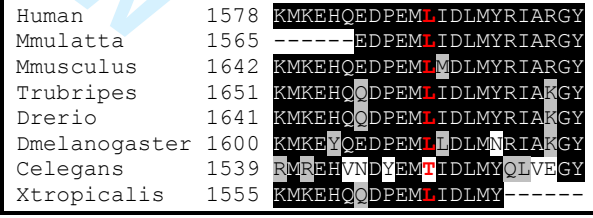
Supp. Table S5: Prediction of pathogenicity and conservation of *DOCK6* unclassified missense variants. (*continued*)

Nucleotide alteration	Predicted effect	PolyPhen-2 HumVar [sensitivity/specificity]	SIFT [Score/Median information content]	MutPred	GERP	Alignment (numbers indicate position of mutated protein)
c.2104G>A	p.G702S	benign (0.211) 0.88 / 0.74	damaging 0.02 / 2.74	0.389	3.62	
c.2767G>A	p.V923I	benign (0.029) 0.94 / 0.59	tolerated 0.12 / 2.71	0.327	3.53	
c.3913C>T	p.R1305C	benign (0.259) 0.87 / 0.75	damaging 0.00 / 2.71	0.559	2.50	
c.4732C>T	p.L1578F	possibly damaging (0.453) 0.83 / 0.80	damaging 0.02 / 2.71	0.594	4.21	

Legend: see above (Supp. Table S2).

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