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ABSTRACT

Background

Sensenbrenner syndrome is a ciliopathy that is characterized by skeletal and ectodermal anomalies, accompanied by chronic renal failure, heart defects, liver fibrosis and other features.

Methods

SNP array analysis and standard sequencing techniques were applied to identify the causative gene. The effect of the identified mutation on protein translation was determined by western blot analysis. Antibodies against intraflagellar transport (IFT) proteins were used in ciliated fibroblast cell lines to investigate the molecular consequences of the mutation on ciliary transport.

Results

We performed homozygosity mapping and positional candidate gene sequence analysis in two siblings with Sensenbrenner syndrome of a consanguineous Moroccan family. In both siblings, we identified a homozygous mutation in the initiation codon of *C14ORF179*. *C14ORF179* encodes IFT43, a subunit of the intraflagellar transport complex A (IFT-A) machinery of primary cilia. Western blots revealed that the mutation disturbs translation of IFT43, inducing the initiation of translation of a shorter protein product from a downstream ATG. The IFT-A protein complex is implicated in retrograde ciliary transport along axonemal microtubules. We show that in fibroblasts of one of the by Sensenbrenner affected siblings, disruption of IFT43 disturbs this transport from the ciliary tip to its base. As anterograde transport in the opposite direction apparently remains functional, the intraflagellar transport complex B proteins accumulate in the ciliary tip. Interestingly, we obtained similar results

using fibroblasts from a Sensenbrenner syndrome patient with mutations in *WDR35/IFT121*, encoding another IFT-A subunit.

Conclusions

Our results indicate that Sensenbrenner syndrome results from disrupted IFT-A mediated retrograde ciliary transport.

INTRODUCTION

Sensenbrenner syndrome or cranioectodermal dysplasia (CED; MIM 218330) is a rare autosomal-recessive heterogeneous ciliopathy that is primarily characterized by skeletal abnormalities (e.g. craniosynostosis, narrow rib cage, short limbs, brachydactyly) and ectodermal defects.[1, 2] Nephronophthisis leading to progressive renal failure, hepatic fibrosis, heart defects, and retinitis pigmentosa have also been described.[3-6] Recently, mutations in two genes have been found to cause Sensenbrenner syndrome, *IFT122* (encoding intraflagellar transport protein 122; MIM 606045) and *WDR35* (encoding WD repeat-containing protein 35; MIM 613602).[7, 8] Both genes encode proteins that are part of the intraflagellar transport (IFT) complex A (IFT-A). This multisubunit complex, consisting of at least six proteins, forms the core of a particle that is mobilized by the cytoplasmic dynein/dynactin motor. The IFT-A particles use the microtubule bundles of the ciliary axoneme to carry membrane vesicles and other "cargo" proteins from the tip of a cilium to its base (retrograde IFT).[9, 10] Both retrograde and anterograde IFT (transport towards the ciliary tip), are required for ciliary assembly, disassembly and homeostasis.[9-11]

METHODS

SNP array analysis

Genomic DNA was isolated from peripheral blood samples of two siblings with Sensenbrenner syndrome and their parents. Each DNA sample was genotyped with an Affymetrix 250K *Nsp*I array (Affymetrix, Santa Clara, CA, USA), which contains 262,000 SNPs. Array experiments were conducted according to protocols from the manufacturer. The 250K SNP genotypes were analyzed with Genotyping Console

software (Affymetrix). PLINK[12] (available online) was used for calculation of regions of homozygosity.

Mutation analysis

Candidate genes were sequenced using patient genomic DNA. Primers for *C14ORF179* and *TTC8* were designed with the Primer 3 program (freely available online); primer sequences are listed in Supplemental Table 3. All coding exons of *C14ORF179* and *TTC8* were amplified by PCR and analyzed forward and reverse with a dye-termination chemistry (BigDye Terminator, version 3 on a 3730 DNA analyzer; Applied Biosystems). PCR conditions are available upon request.

DNA constructs

A full length cDNA clone (IRAUp969A1256 from ImaGenes) was used as a template to amplify full length *C14ORF179* (NM_052873, isoform 1) with Gateway compatible primers. A consensus kozak sequence "gccgccacc" or a *C14ORF179* 5'UTR fragment "gtttccaggaagtgacgtcaggcggccgcggag" was included in the forward primers, just prior to the "atg". PCR products were used to create Gateway-adapted constructs by using the Gateway cloning system (Invitrogen). *C14ORF179* was then cloned into two vectors, the Gateway-adapted SF-TAP vector (with a C-terminal Strep II-FLAG-tag)[13] and p504 (with a C-terminal eYFP tag).[14] Wild type constructs and vectors containing the translation initiation codon mutation were made.

Transfection and western blot analysis

HEK293T cells (human embryonic kidney) were transfected with plasmid DNA using effectene (Qiagen) in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal

Calf Serum (FCS) according the manufacturer's instructions. 24 hours after transfection, cells were lysed in ice cold lysis buffer (1x TBS, 0,5% NP-40 with complete protease inhibitor cocktail, Roche). Lysates containing IFT43-eYFP and IFT-Strep II-FLAG were cleared by centrifugation at 4 °C for 10 minutes at 14,000 g. After adding NuPAGE sample buffer with reducing agent (Invitrogen) to the samples, lysates were heated for 10 minutes at 70 °C. Samples were subsequently analyzed by SDS-PAGE (by using a NuPAGE Novex 4%-12% Bis-Tris SDS-PAGE gel) followed by western blotting using α-FLAG (Flag M2 mouse monoclonal, Sigma, 1:1000) and α-GFP (GFP mouse monoclonal, Roche, 1:1000) antibodies. IRDye800 (Rockland) goat anti-mouse IgG was used as a secondary antibody (1:10,000). Blots were washed with PBS with 0.1% Tween-20. Fluorescense was detected on a Li-Cor Odyssey 2.1 infrared scanner.

Immunocytochemistry

Fibroblast cells from Sensenbrenner patients (CL10-00031 and CL10-00021) and a control individual (CL10-00010) were stained with antibodies to the following proteins: IFT88 and IFT57 (rabbit polyclonal; both kindly provided by G. Pazour; 1:300 and 1:250, respectively); GT335 (mouse monoclonal; kindly provided by C. Janke; 1:1500); RPGRIP1L (guinea pig polyclonal; SNC040, 1:500),[14] and acetylated alpha-tubulin (mouse monoclonal, Zymed laboratories, 1:1000). The secondary antibodies (all from Molecular Probes) that were used are as following: anti-guinea pig IgG Alexa Fluor 568 and 488 (1:300), anti-rabbit IgG Alexa Fluor 568 and 488 (1:300) and anti-mouse IgG Alexa Fluor 405 (1:300). Fibroblast cells were seeded (~1:3) on sterile cover glasses and incubated for 24 hours at standard cell culture conditions in Dulbecco's modified Eagle's medium (DMEM) with 20% Fetal

Calf Serum (FCS). Cells were subsequently serum-starved for 48 hours with DMEM with 0,2% FCS to stimulate cilia formation. Cells were then briefly washed in PBS and fixed in 2% paraformaldehyde in PBS for 20 minutes. Cover glasses were subsequently blocked with freshly made 2% BSA in PBS, followed by a 1 hour incubation with the primary antibody at room temperature. Cells were washed with PBS and then incubated with secondary antibodies for 30 minutes. Cover glasses with the stained cells were placed upside-down on a drop of Vectashield (Vector Laboratories) on a microscopic glass slide. Microscopic analysis was performed on a Zeiss Axio Imager Z1 fluorescene microscope (Zeiss) with an ApoTome slider. Images were processed with AxioVision (Zeiss) and Photoshop CS4 (Adobe Systems).

Accession numbers and web resources

Accession numbers of the *C14ORF179* gene and a list of the web resources that were used in this study can be found in Supplementary Materials and Methods.

RESULTS

Clinical studies and genotyping

To identify additional genetic defects underlying Sensenbrenner syndrome, we conducted genome-wide homozygosity mapping by using Affymetrix 250K arrays in a consanguineous family from Moroccan descent with two siblings with Sensenbrenner syndrome (family P05-1040, Figure 1 and Supplemental Table 1). Homozygosity analysis by using PLINK[12] revealed that the largest- and second-largest homozygous regions of the affected siblings from family P05-1040

(individuals II:1 and II:2, Table 1) are overlapping; this region was 25.2 Mb on chromosome 14 (containing 158 annotated RefSeq genes), with SNP_A-2107558 and SNP_A-2312334 as bordering SNPs (Figure 1, Table 1 and Supplementary Table 2). Other homozygous regions shared less overlap, indicating that the genetic defect was most likely to be found in the region on chromosome 14 that contains the genes *C14ORF179* and *TTC8*.

Given that *IFT122* and *WDR35*, which are both associated with Sensenbrenner syndrome, are part of the IFT-A protein complex, we selected *C14ORF179* as our primary candidate, as this gene encodes the human orthologue of another member of the IFT-A complex, IFT43.[9, 10] In addition, we selected *TTC8* (tetratricopepteide repeat domain 8; MIM 608132) as a candidate gene. This gene encodes an IFT regulator and is disrupted in Bardet-Biedl syndrome (BBS; MIM 209900).[15, 16]

Mutation detection

Sequence analysis of candidate genes *TTC8* and *C14ORF179* in the affected siblings excluded genetic defects in *TTC8* as a cause of Sensenbrenner syndrome in these patients, but did reveal a homozygous variation in the translation initiation codon in exon 1 of *C14ORF179* (c.1A>G) (Figure 2A). This variation cosegregates with the disorder in the family, as both (related) parents are heterozygous for this variation (Figure 2A). The c.1A>G variant was neither present in dbSNP, nor in 192 Dutch- and 122 Moroccan control alleles, respectively, which provides further evidence for the pathogenicity of the identified mutation.

In an attempt to identify more mutations in *C14ORF179*, we screened this gene for mutations in four unrelated Sensenbrenner patients who did not carry *WDR35* mutations. We did not identify mutations in these patients. Since homozygosity

mapping in patients from two consanguineous Sensenbrenner families did not reveal major stretches of homozygosity in the regions containing *WDR35*, *IFT122* or *C14ORF179*, it is likely that the heterogeneity in Sensenbrenner syndrome extends beyond these three genes.

C14ORF179 mutation causes aberrant translation of the encoded IFT43 protein

C14ORF179 contains 10 exons that encode two major protein isoforms derived from alternative splicing that only vary in a central segment of both isoforms (Figure 2B-C). Although ATG>GTG mutations have been reported to be pathogenic (e.g. in hereditary osteodystrophy,[17] Norrie disease,[18] and beta-thalassemia[19]), it has also been described that the GTG codon can be used as an initiation codon, albeit rare and inefficient.[20-22] We therefore investigated the effect of the c.1A>G mutation in *C14ORF179* on its encoded protein. To test if this mutation diminishes translation or if it leads to translation initiation from a downstream ATG (most likely from the first downstream ATG in exon 2), we cloned the full length *C14ORF179* cDNA with and without the c.1A>G mutation into expression vectors encoding eYFP- and Strep II-FLAG fusion tags. Western blot analysis of the recombinant tagged IFT43 proteins reveals that the translation initation codon mutation indeed disturbs translation; introduction of the mutation yields IFT43 proteins with a lower molecular weight of ~3 kDa compared to wild type proteins (Figures 2D and 2E). This is in agreement with translation initiation at the second ATG of the coding sequence (in exon 2), at position c.A64, that becomes the first available initiation codon due to the mutation. This results in an N-terminal deletion of 21 amino acids (p.Met1_Val21del) in the same open reading frame.

Mutation in *C14ORF179*/IFT43 disrupts ciliary transport

The structure of IFT43, the protein product of *C14ORF179*, remains largely elusive as the protein contains no significant homology with any structural or functional motifs (as determined by using SMART[23]) (Figure 2C). The protein was initially studied in *Chlamydomonas reinhardtii*.[9] The *Chlamydomonas* orthologue is part of the intraflagellar transport complex A that is involved in retrograde transport in the cilium.[9, 10] The IFT-A protein complex consists of IFT43, IFT121/WDR35, IFT122, IFT139, IFT140 and IFT144[10], and is associated to THM1 and TULP3.[24] Recently, it was shown that IFT43 directly binds to IFT121/WDR35, which supports a tight relationship between these two proteins.[25] This is remarkable, since IFT121/WDR35 is the protein product of *WDR35*, a previously identified Sensenbrenner gene.[8]

Studies in multiple species, including *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Trypanosoma brucei*, have shown that disruption of retrograde transport leads to formation of bulged cilia that contain an accumulation of IFT-B complex proteins in their tips.[24, 26-30] In order to validate the disruptive effect of the identified mutation in *C14ORF179* in family P05-1040 (Figures 1 and 2), we stained fibroblasts from one of the affected siblings (II:2) using antibodies against the IFT-B complex protein members IFT88 and IFT57. Indeed, we identified a similar accumulation of IFT-B complex proteins in the distal part of the ciliary axoneme and in the ciliary tip (Figure 3 and Supplementary Figures 1 and 2), while in cilia of control fibroblasts, these proteins were less abundant and primarily localized at the basal body and transition zone.

Subsequently, we compared these ciliary defects with the phenotype of cilia in fibroblasts from a Sensenbrenner patient with *WDR35* mutations (a patient that we described previously[8]), and found nearly identical defects (Fig 3 and Supplementary Figures 1 and 2). Moreover, similar to what was reported for Sensenbrenner patients with *IFT122* mutations,[7] we found that cilia from patient fibroblasts (II:2, Figure) are somewhat shorter than those of healthy controls (Supplementary Figure 3).

DISCUSSION

We report on a family with two children with Sensenbrenner syndrome who carry a mutation in the translation initiation codon of *C14ORF179*. This is the third gene associated with Sensenbrenner syndrome and in line with the other two genes (*WDR35* and *IFT122*), *C14ORF179* also encodes a member of the IFT-A particle (IFT43). Mutations in *WDR35* can cause both Sensenbrenner syndrome and the more severe (and embryonically lethal) Short Rib-Polydactyly (personal communication, P.J. Lockheart). We therefore suggest that both syndromes are part of a phenotypic spectrum of IFT-A complex disruption, similar to what has been proposed for Joubert syndrome and allied ciliopathies like Meckel-Grüber syndrome and Bardet-Biedl syndrome. The remaining protein activity then determines the severity of the disease phenotype. Indeed, in all genes associated with Sensenbrenner syndrome identified thus far, only mutations with a relatively mild disruptive character (mainly missense mutations, sometimes in combination with a truncating mutation) were identified, including the mutation described in this report which results in a small, N-terminal truncation of IFT43. In this respect, it is interesting to note that although we show that such mild mutations in cilia of Sensenbrenner patients' fibroblasts disrupt retrograde transport, they do not abrogate ciliogenesis.

Sensenbrenner syndrome clinically overlaps with Jeune syndrome (also known as Asphyxiating Thoracic Dystrophy; ATD; MIM 208500). Skeletal anomalies such as brachydactyly, short limbs and a narrow thorax have been reported in both disorders. A skeletal characteristic that is unique for Sensenbrenner syndrome is craniosynostosis, however, only the youngest sibling in family P05-1040 displayed this typical characteristic (Supplementary Table 1, patient II:2). This complicated the initial clinical diagnosis in this family as it was unclear whether this family suffered from Sensenbrenner syndrome or a mild form of Jeune syndrome.

Like in Sensenbrenner syndrome, genes associated with Jeune syndrome encode proteins involved in ciliary transport. Mutations have been identified in *IFT80* (intraflagellar transport 80; MIM 611177) that encodes an IFT-B complex protein, as well as in *DYNC2H1* (dynein, cytoplasmic 2, heavy chain 1; MIM 603297) which is a retrograde motor transporting the IFT-A particle.[31, 32] Because of the clinical- and functional overlap between Sensenbrenner and Jeune syndrome, we also performed mutation analysis in 17 Jeune patients. Although we did not detect any *C14ORF179* mutations in these patients, which indicates that this is not a frequent cause of Jeune syndrome, it remains a functional candidate gene.

In conclusion, we identified a homozygous mutation in the translation initiation codon in *C14ORF179* that causes Sensenbrenner syndrome in a consanguineous family of Moroccan descent. The identified genetic defect interferes with translation, resulting in a shortened protein. Consistent with the disruption of a member of the IFT-A protein complex, fibroblasts from one of the affected siblings (II:2) show a typical IFT-A defect (i.e. accumulation of IFT-B complex proteins in the ciliary tip). Our

results demonstrate that Sensenbrenner syndrome results from defects in retrograde intraflagellar transport.

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Ethics Approval

This study was approved by the Medical Ethics Committee of the Radboud University Nijmegen Medical Centre.

Informed Consent

Obtained. This includes written informed consent from the parents of the patients (II:1 and II:2) for publication of the images.

Competing interests

None.

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Figure 1. Family P05-1040 is affected by Sensenbrenner syndrome. (A) P05-1040 is a consanguineous family from Moroccan descent with two siblings with Sensenbrenner syndrome. Clinical features of patient II:1 (B-F) and patient II:2 (G-K). (B) Absence of typical craniofacial features. (C) Rhizomelic shortening of limbs, narrow thorax. (D) Hypoplastic, cone shaped, and widely spaced teeth. (E) Bilaterial 2-3-4 toe syndactyly. (F) Brachydactyly, webbing of fingers, short and broad nails. (G) Frontal bossing, telecanthus, micrognathia, sparse and fine hair. (H) Rhizomelic shortening of limbs, narrow thorax. Haemodialysis catheter, Ciminoshunt left arm, gastrostomy. (I) Hypoplastic and widely spaced teeth. (J) Bilateral postaxial polydactyly, bilateral 2-3 toe and 5-6 toe syndactyly, sandal gap between 1-2 toes. (K) Brachydactyly, webbing of fingers, short and broad nails after surgical correction of postaxial polydactyly. Written informed consent from the parents was obtained to publish images from patients II:1 and II:2.

Figure 2. Mutations in *C14ORF179* cause Sensenbrenner syndrome. (A) In family P05-1040, both affected siblings (II:1 and II:2, left panel) carry a homozygous mutation (c.1A>G) in the translation initiation codon of *C14ORF179*. Both first cousin parents (I:1 and I:2, right panel) are heterozygous for this variation. The asterisk shows the reference sequence. (B) *C14ORF179* contains 10 exons and is alternatively spliced. The mutation (c.1A>G) that was identified in family P05-1040 as a cause of Sensenbrenner syndrome is indicated with an asterisk. (C) Two major splice variants of *C14ORF179* encode two different isoforms of IFT43 that vary in their central protein domain. (D-E) Western blot analysis of cell lysates from HEK293 cells expressing recombinant wildtype (lanes 1 and 2) and mutated IFT43 (lane 3), C-terminally fused with a Strep II-FLAG tag (left panel) or an eYFP tag (right panel). Variations of the initiation codon and upstream sequence of *C14ORF179* are indicated. The initiation codon mutation interferes with translation, resulting in a shortened protein (lane 3). The difference between the mutated and the wild type protein is approximately ~3 kDa in both α-FLAG and α-GFP immunoblots (compare lanes 2 and 3). A wildtype construct with a kozak sequence (instead of the ‘5 UTR fragment of *C14ORF179*) was used as a control for protein expression.

Figure 3. IFT88 and IFT57 accumulate in distal ends of cilia in fibroblasts from Sensenbrenner patients with *C14ORF179* and *WDR35* mutations. A) Fibroblasts were stained against the IFT-B complex protein IFT88, GT335 (a marker of the proximal cilium) and RPGRIP1L (a transition zone marker). IFT88 (green); GT335 (purple); and RPGRIP1L (red). Comparison of the IFT88 staining in control fibroblasts with cells from Sensenbrenner patients reveals that IFT88 accumulates in the ciliary tips of the patient's fibroblasts. Note, that there is a certain extent of variability between IFT-defects in cilia from the patients; i.e. in some cilia the IFT88 staining is more intense (and more distal) than in others. B) Fibroblasts from Sensenbrenner patients with *C14ORF179* and *WDR35* mutations were stained for IFT57 (an IFT-B complex protein), and two markers that stain the base of the cilium, GT335 and RPGRIP1L. IFT57 (green); GT335 (purple); and RPGRIP1L (red). Like IFT88, IFT57 accumulates in the distal ends of cilia in fibroblasts from the patients.

Table 1. Homozygosity mapping in Sensenbrenner family P05-1040

Patient	Chromosome	Bordering SNP (5')	Bordering SNP (3')	Size of region (Mb)	# SNPs in homozygous region	Homozygous region (i.e.1 = largest, 2 = second largest)
II:1	14	SNP_A-2107558	SNP_A-1781135	25.3	2425	1
II:2	14	SNP_A-2107558	SNP_A-2312334	25.2	2398	2
			overlap	25.2		
II:1	2	SNP_A-2123667	SNP_A-1865387	2.7	407	11
II:2	2	SNP_A-1959026	SNP_A-2248948	18.8	2130	5
			overlap	2.6		
II:1	11	SNP_A-1895498	SNP_A-4197277	6.0	271	6
II:2	11	SNP_A-2268220	SNP_A-4197277	6.0	276	11
			overlap	6.0		
II:1	11	SNP_A-4204835	SNP_A-2142191	4.1	408	9
II:2	11	SNP_A-4204835	SNP_A-4205202	4.1	407	14
			overlap	4.1		
II:1	3	SNP_A-1923781	SNP_A-2252922	1.4	95	18
II:2	3	SNP_A-1820067	SNP_A-1974247	5.1	394	13
			overlap	1.4		
II:1	3	SNP_A-1972308	SNP_A-1829330	1.1	108	24
II:2	3	SNP_A-1972309	SNP_A-1972369	2.0	209	17
			overlap	1.0		
II:1	17	SNP_A-2206444	SNP_A-1872313	1.1	71	23
II:2	17	SNP_A-2302032	SNP_A-2156489	1.1	73	23
			overlap	1.1		
II:1	5	SNP_A-4210912	SNP_A-4202173	1.0	54	28
II:2	5	SNP_A-2084301	SNP_A-2293403	1.1	64	22
			overlap	1.0		

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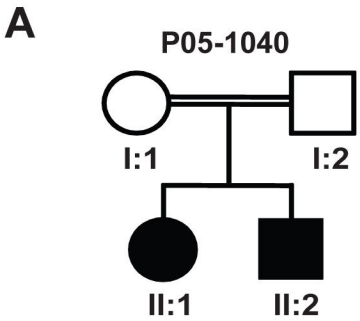
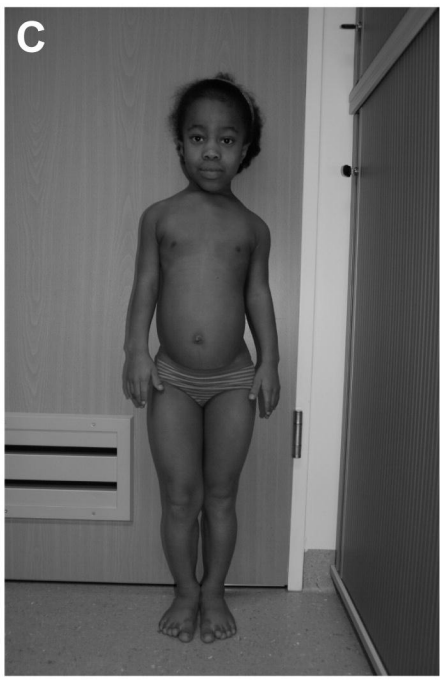
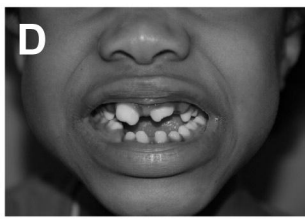
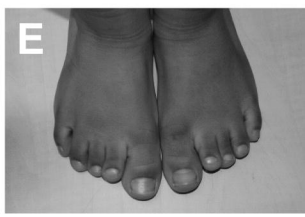
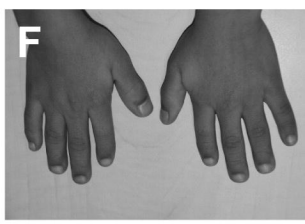
Figure 1

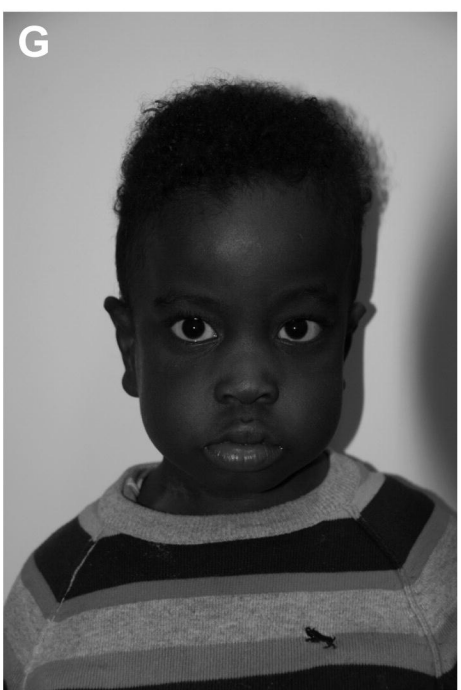


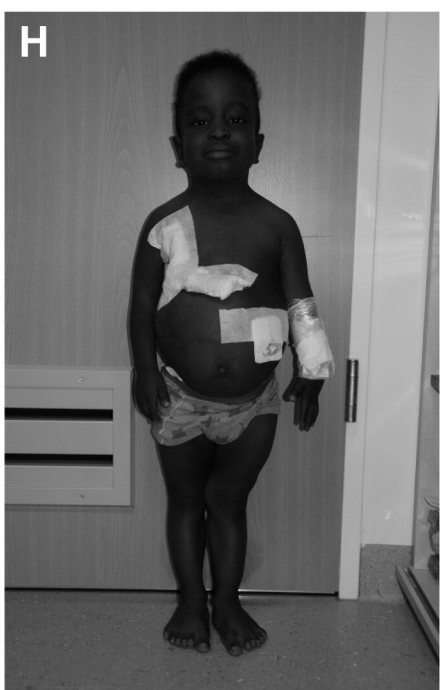


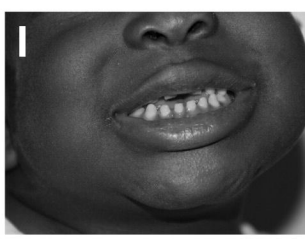


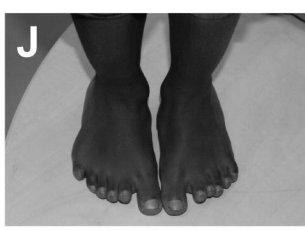












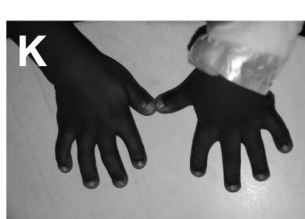
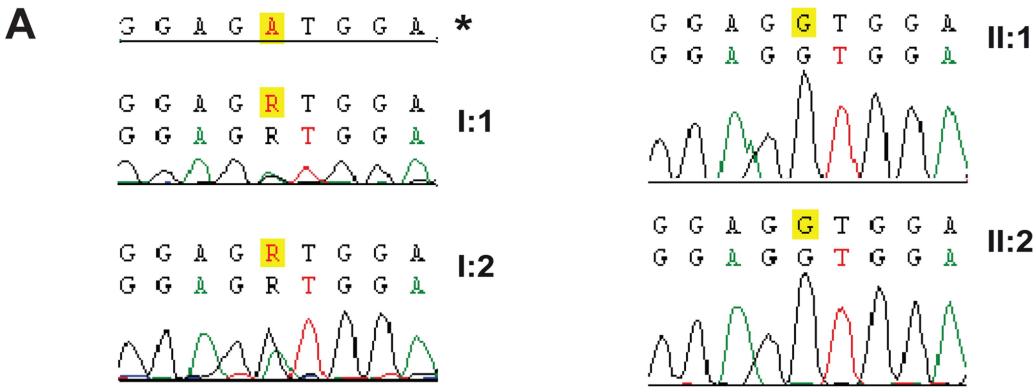


Figure 2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II:1
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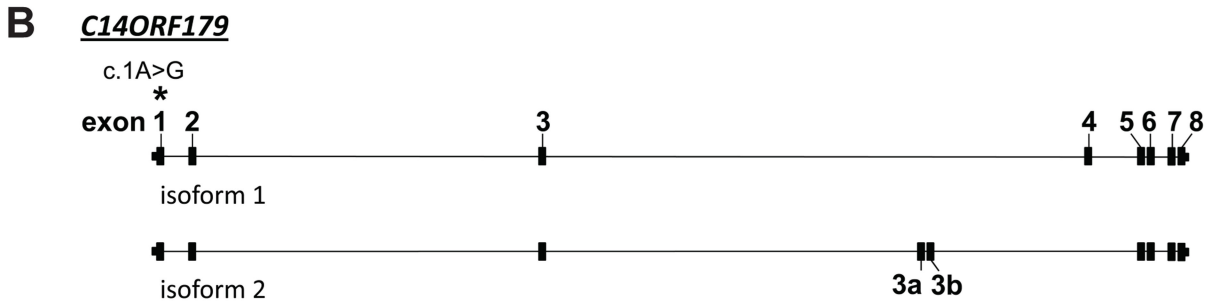
G G A G G T G G A
II:2


B
C14ORF179
c.1A>G
*
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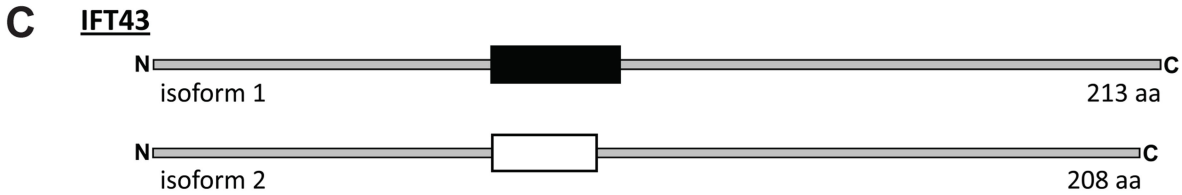
isoform 1
isoform 2
3a 3b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IFT43
N
C
isoform 1
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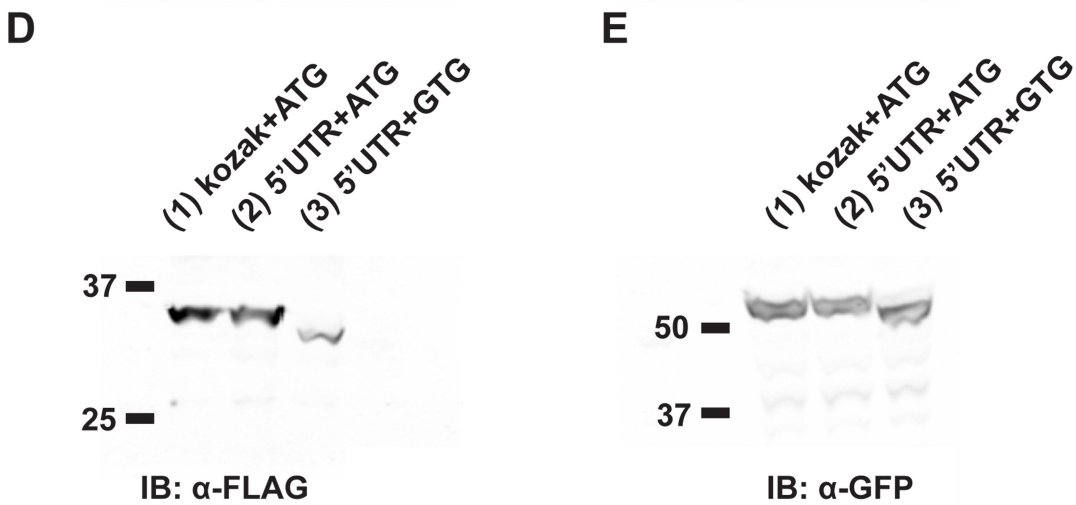
N
C
isoform 2
208 aa


D
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(2) 5'UTR+ATG
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37
25
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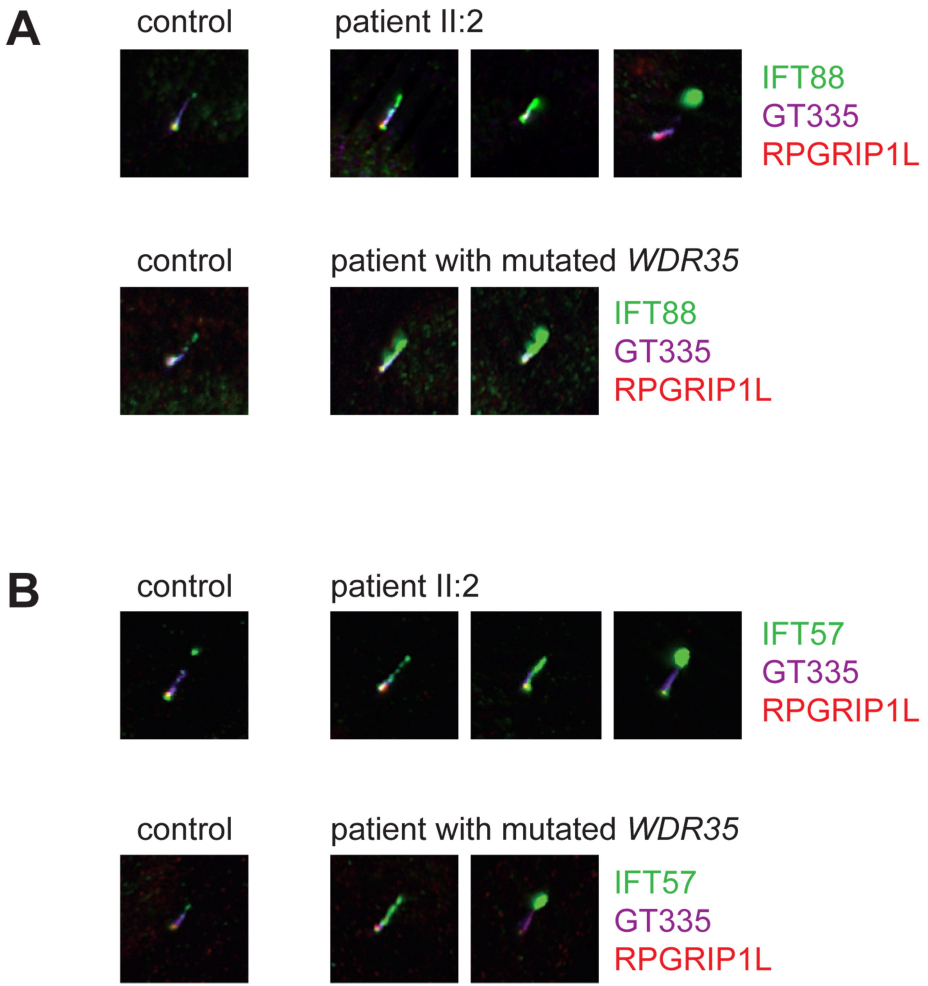
50
37
IB: α-GFP

Figure 3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IFT57
GT335
RPGRIP1L
control
patient with mutated *WDR35*
IFT57
GT335
RPGRIP1L