Mutations in \textit{DYNC2LI1} disrupt cilia function and cause short rib polydactyly syndrome

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The short rib polydactyly syndromes (SRPSs) are a heterogeneous group of autosomal recessive, perinatal lethal skeletal disorders characterized primarily by short, horizontal ribs, short limbs and polydactyly. Mutations in several genes affecting intraflagellar transport (IFT) cause SRPS but they do not account for all cases. Here we identify an additional SRPS gene and further unravel the functional basis for IFT. We perform whole-exome sequencing and identify mutations in a new disease-producing gene, cytoplasmic dynein-2 light intermediate chain 1, \textit{DYNC2LI1}, segregating with disease in three families. Using primary fibroblasts, we show that DYNC2LI1 is essential for dynein-2 complex stability and that mutations in \textit{DYNC2LI1} result in variable length, including hyperelongated, cilia, Hedgehog pathway impairment and ciliary IFT accumulations. The findings in this study expand our understanding of SRPS locus heterogeneity and demonstrate the importance of DYNC2LI1 in dynein-2 complex stability, cillum function, Hedgehog regulation and skeletogenesis.
The primary cilium is a microtubule-based projection on the
surface of nearly every cell that acts as a concentrated
signalling centre for several pathways. Defects in the
formation or function of primary cilia cause a wide variety of
diseases known as ciliopathies. As a testament to the importance
of this organelle, ciliopathies collectively affect all major organ
systems, including the skeleton. Cilium assembly and function
require intraflagellar transport (IFT), a system for bidirectional
traffic into and out of this organelle. IFT depends on the
microtubule motor protein activities of two multi-protein
complexes: IFT-B, the kinesin-2-driven antero-gradecomplex,
mediates base-to-tip transport, and IFT-A, the dynein-2-driven
retrograde complex, mediates tip-to-base transport.

The SRPSs are autosomal recessive, perinatal lethal disorders.
Radiographic abnormalities include very short, horizontal ribs,
short limbs and variable degrees of polydactyly. Other
organ system abnormalities, particularly those affecting the
heart and kidney, are frequently seen. SRPSs are part of
a spectrum of heterogeneous skeletal ciliopathies that
include asphyxiating thoracic dystrophy, Ellis-van Creveld and
Sensenbrenner syndromes. Mutations in the gene encoding
the IFT-A cytoplasmic dynein-2 motor heavy chain, DYNC2H1
(MIM 603297), are the most common cause of both SRPS and
asphyxiating thoracic dystrophy. Recently, mutations in
several additional genes have been shown to cause SRPS: IFT80
(refs 6,7; MIM 611263), WDR35(refs 8,9; MIM 614091),
WDR19 (ref. 10; MIM 614376), NEK1 (refs 11,12; MIM 263520),
WDR34 (refs 13,14; MIM 615633), IFT140 (refs 15,16; MIM
266920), IFT172 (ref. 17; MIM 615630), WDR60 (ref. 18;
MIM 615462) and TTC21B (refs 19,20; MIM 612014). Among the proteins
encoded by the SRPS genes, WDR60 (FAP153; ref. 21), the light intermediate
chain DIC5/FAP133; ref. 22) and WDR34 (DIC5/FAP133; ref. 23)
are targeted to the non-ciliary
components: the dynein heavy chain DYNC2H1 (refs 24,25). Finally, the other
intermediate chains WDR34 (DIC5/FAP133; ref. 22) and
WDR60 (FAP153; ref. 21) interact with the dynein-2 complex; the more closely related
motor to substrates such as vesicles and providing docking
sites for cargo. It is essential for ciliogenesis and contains an
X-box promoter that is recognized by the ciliary transcription
factor RFX3/DAF-19 (refs 26,27). Recent studies have shown
that DYNC2LI1 is essential for dynein-2 complex stability and its depletion in
the reed-macallum light intermediate chain DYC2H1
(D1bLIC/D2LIC/LIC3/XBX1; refs 26–29) and the
LC8 light chain. DYNC2LI1 was described as a novel dynein
light intermediate chain based on its amino acid sequence, its
ability to interact with DYNC2H1 and its localization to cilia.
As an accessory chain binding to the base of the dynein-2
complex, DYNC2LI1 is likely involved in tethering the dynein-2
motor to substrates such as vesicles and providing docking
sites for cargo.

In this study, we identify mutations in DYNC2LI1 causing
SRPS in three independent cases. We show that DYNC2LI1 is
essential for dynein-2 complex stability, is expressed in the
cartilage growth plate and plays critical roles in the regulation
of primary cilium length, retrograde IFT and Hedgehog signalling.
Analysis of SRPS-derived fibroblasts supports a role for
DYNC2LI1 in cilia function and signalling and the findings
highlight the importance of this gene and the dynein-2 complex
in skeletogenesis.

**Results**

**DYNC2LI1 mutations identified in SRPS cases.** To identify
additional genes that are essential for skeletal development, we
used whole-exome sequencing under an approved human
subjects protocol and identified mutations in the dynein-2
light intermediate chain gene, DYNC2LI1, producing SRPS.
Three probands from unrelated Caucasian families were
identified by prenatal ultrasound, and elective terminations were
performed at 14, 19 and 22 weeks, respectively. In the prenatal
period, ultrasound findings included shortened long bones,
diminished chest circumferences for gestational age and poly-
dactyly. No other obvious organ system abnormalities were
detected by ultrasound. Radiographic analyses showed poly-
dactyly of the upper and lower extremities, a long narrow thorax
with very short horizontal ribs, lack of ossification of some
skeletal elements and irregular metaphyseal borders with lateral
spikes (Fig. 1). These findings are characteristics of SRPS and
radiographically similar to SRPS cases described with DYNC2H1
and WDR34 mutations.

In all three cases there was compound heterozygosity for
mutations in DYNC2LI1 (Supplementary Fig. 1). Two affected
individuals, R01-013A and R03-303A, were heterozygous for
changes in intron 12 (c.993 +1G>A, c.993 +3A>G) that
are predicted to affect splicing (Table 1). RT–PCR (PCR
with reverse transcription) analysis on RNA derived from
fibroblasts from both fetuses demonstrated that both mutations
resulted in in-frame skipping of exon 12 (Fig. 2a). R01-013A
and R07-628A were both heterozygous for a point mutation
causing the predicted amino acid p.Leu117Val substitution.
This missense mutation occurs within a region conserved among
the three mammalian dynein light intermediate chains and
resides within an NTPase-related domain (Supplementary Fig. 2).
This mutation may interfere with cargo-binding and occurs within a homologous region that is required for
cargo-binding in DYNCL1 (ref. 37). However, of the three
mammalian dynein light intermediate chains, only DYNC2LI1
interacts with the dynein-2 complex; the more closely related
DYNC1LI1 and DYNC1LI2 are targeted to the non-ciliary
complexes of the dynein-2 intermediate chain WDR34 and the heavy chain
DYNC2H1 mutations in SRPS cases

**DYNC2LI1-mutant fibroblasts are depleted of DYNCL1.**
DYNC2H1 and DYNC2LI1 are distinct components of the ciliary
dynein-2 complex, and studies in C. reinhardtii and T. brucei
show that loss of one results in loss or reduction of the
other. We determined whether DYNC2H1 amounts were
affected by the decrease in DYNC2LI1, we probed whole-cell lysates from SRPS cases and controls for DYNCL1 and
found near-total loss of the protein (Fig. 3a). The amounts of the
light intermediate chain WDR34 and the heavy
chain of the dynein-1 complex (DYNC1H1) were unchanged,
indicating that the effects of the DYNC2LI1 mutations
were specific to DYNCL1. Immunofluorescence staining showed
that WDR34 and the minimal remaining DYNCL1 in SRPS cells
localized to the basal bodies, similar to controls (Fig. 3b).
These results suggest that even though DYNC2LI1 is important
for the stabilization of DYNCL1, it is not essential for the
recruitment of the dynein-2 complex to the pericentriolar region of basal bodies.
DYNC2LI1 is expressed in the developing human growth plate. While the dynein-2 complex is required for IFT in all ciliated cells of the body, the marked skeletal consequences of dynein-2 complex mutations suggest that the developing skeleton is very sensitive to reductions in its activity. Concordant with this hypothesis, transcriptional analysis by quantitative RT–PCR (qRT–PCR) showed that DYNC2LI1 was more highly expressed in bone compared with other embryonic tissues (bone, brain, cartilage, heart, liver, lung, placenta and thymus; Supplementary Fig. 3). Furthermore, immunohistochemistry of human cartilage growth plate revealed DYNC2LI1 expression in the perichondrium, periosteum and primary spongiosa of bone (Fig. 4). Similarly, immunohistochemistry of DYNC2H1 and WDR34 showed an overlapping expression pattern (Fig. 4). Together, these expression studies support a role for DYNC2LI1 and the dynein-2 complex in human skeletal development.

SRPS fibroblasts exhibit loss of ciliary length control. To investigate whether mutations in DYNC2LI1 affect ciliogenesis and/or IFT, we quantified the fraction of ciliated cells, measured cilia length and probed SRPS and control fibroblasts for a number of ciliary components. The percentage of ciliated cells was only slightly reduced between SRPS and control cells (Fig. 5b). However, we found that primary cilia length was highly variable in SRPS fibroblasts, with a substantial increase in the number of hyperelongated cilia up to 20 μm in length (Fig. 5a,c,d).

To determine whether the ciliary length variation observed in SRPS cells resulted from decreased amounts of DYNC2LI1, we performed RNAi against DYNC2LI1 in hTERT–RPE1 (retinal epithelial) cells. DYNC2LI1 knockdown led to significantly increased variation in cilia length, thus reproducing the phenotype observed in SRPS fibroblasts (Supplementary Fig. 4a,b). To test for phenotypic rescue, we expressed wild-type DYNC2LI1 in SRPS cells. As shown in Fig. 5c,d, transfection of DYNC2LI1 into SRPS fibroblasts reduced ciliary length variation and restored length to control values. We therefore conclude that DYNC2LI1 and the dynein-2 complex play an important role in the regulation of primary cilia length.

IFT components accumulate in DYNC2LI1-mutant cilia. The IFT-B components, IFT88 and TRAF3IP1, and the IFT-B kinesin motor, KIF3A, localized strongly to the base of the cilia and

| Sample ID | Genotype | Mutation | Location | Cons* | Sift | Polyphen | CADD† | rsID | AF| |
|---|---|---|---|---|---|---|---|---|---|
| R01-013A | 0/1 | c.993+1G>A | Intron 12 | 4.37 | 3.63 | p.Leu117Val | Exon 6 | 16.1 | | |
| R07-628A | 0/1 | c.349C>G | p.Leu117Val | Exon 6 | 3.63 | 3.63 | Deleterious | 18.6 | rs201948500 | 0.000023 |
| R01-013A | 0/1 | c.993+3A>G | Intron 12 | 4.37 | | 16.1 | | 0.00008 |
| R03-303A | 0/1 | c.349C>G | p.Leu117Val | Exon 6 | 3.63 | 3.63 | Deleterious | 18.6 | rs201948500 | 0.000023 |
| R03-303A | 0/1 | c.372G>A | p.Trp124Ter | Exon 6 | 4.46 | | 26.5 | | |
| R03-303A | 0/1 | c.1000G>T | p.Glu334Ter | Exon 13 | 4.55 | | 38.0 | | |
| R03-303A | 0/1 | c.993+3A>G | Intron 12 | 4.37 | | 16.1 | | 0.00008 |

*Conservation. †Phred-scaled CADD score. ‡Mutant allele frequency.

Figure 1 | Mutations in DYNC2LI1 cause SRPS. Radiographs of the affected probands, R03-303A, R01-013A and R07-628A (International Skeletal Dysplasia Registry reference numbers) showing short long bones, horizontal ribs and long narrow chest, poor mineralization of some skeletal elements (single arrow), metaphyseal irregularity (double arrows) and polydactyly in all the limbs of the affected individuals.
weakly along the axoneme in control cells, as observed by others\(^{39}\) (Fig. 6a). Consistent with defective retrograde transport in SRPS fibroblasts, IFT88, TRAF3IP1 and KIF3A all accumulated near the cilia tip. More specifically, three- to four-fold more IFT88 was retained in the axonemes of \(\text{DYNC2LI1}\)-mutant cilia than in controls (Fig. 6c). RNAi knockdown of \(\text{DYNC2LI1}\) in RPE1 cells replicated this finding by increasing the ratio of IFT88 retained in the primary cilium body relative to the proximal end by threefold (Supplementary Fig. 4c,d). In addition, we were able to reduce this ratio in SRPS fibroblasts by expressing wild-type \(\text{DYNC2LI1}\) (Fig. 6b,c). Our results demonstrate that loss of \(\text{DYNC2LI1}\) markedly impairs retrograde IFT and leads to the accumulation of IFT proteins within the primary cilium.

**Hedgehog signalling requires intact cilia and IFT; not surprisingly, many IFT mutants display defects in this pathway\(^{40}\). To determine whether \(\text{DYNC2LI1}\) mutations affect Hedgehog signalling, we examined the localization of smoothened, frizzled class receptor (SMO) and GLI3 in SMO agonist (SAG)-stimulated and -unstimulated fibroblasts from SRPS cases and controls. While the localization of GLI3 did not differ between SRPS and control cells (Fig. 7a), we detected SMO along the length of cilia of unstimulated \(\text{DYNC2LI1}\)-mutant cells (Fig. 7b,c). In contrast, SMO was only observed in the cilia of control cells upon stimulation. Similar to findings in \(\text{DYNC2H1}\) mouse mutants\(^{41}\), these observations suggest that \(\text{DYNC2LI1}\) and the activity of the dynein-2 complex play an important role in preventing SMO from entering cilia in the absence of Hedgehog stimulation.

**Figure 2 | Effect of \(\text{DYNC2LI1}\) mutations on splicing and protein stability.**

(a) RT-PCR of cDNA derived from control and SRPS fibroblasts shows that \(\text{DYNC2LI1}\) splice-donor mutations cause in-frame skipping of exon 12. (b) Immunoblotting reveals significant reduction in the amounts of \(\text{DYNC2LI1}\) from lysates of affected cells compared with control. Size markers (right, kDa), \(\gamma\)-tubulin, loading control. (c) Quantification of average \(\text{DYNC2LI1}\) amounts from (b) for each genotype. Values were normalized to control and error bars represent ± s.e.m (three independent experiments). Statistical analyses were performed using the Mann-Whitney test, \(* P<0.05.\)**

**Figure 3 | Mutations in \(\text{DYNC2LI1}\) decrease the stability of \(\text{DYNC2H1}\).**

(a) Immunoblotting for components of the dynein-2 complex (heavy chain, \(\text{DYNC2H1}\); intermediate chain, \(\text{WDR34}\)) and the heavy chain of the dynein-1 complex (\(\text{DYNC1H1}\)) reveals a specific reduction of \(\text{DYNC2H1}\). (b) Immunofluorescence micrographs of control and SRPS cells stained for GluTUB (red) and \(\text{WDR34}\) (green) or \(\text{DYNC2H1}\) (green). Cilia (2 \(\times\)) are shown on the right. Scale bar, 5 \(\mu\)m.

**SMO inappropriately enters the cilia of SRPS fibroblasts.** Hedgehog signalling requires intact cilia and IFT; not surprisingly, many IFT mutants display defects in this pathway\(^{40}\). To determine whether \(\text{DYNC2LI1}\) mutations affect Hedgehog signalling, we examined the localization of smoothened, frizzled class receptor (SMO) and GLI3 in SMO agonist (SAG)-stimulated and -unstimulated fibroblasts from SRPS cases and controls. While the localization of GLI3 did not differ between SRPS and control cells (Fig. 7a), we detected SMO along the length of cilia of unstimulated \(\text{DYNC2LI1}\)-mutant cells (Fig. 7b,c). In contrast, SMO was only observed in the cilia of control cells upon stimulation. Similar to findings in \(\text{DYNC2H1}\) mouse mutants\(^{41}\), these observations suggest that \(\text{DYNC2LI1}\) and the activity of the dynein-2 complex play an important role in preventing SMO from entering cilia in the absence of Hedgehog stimulation.
DYNC2LI1 mutants exhibit reduced GL3 processing. To further investigate Hedgehog pathway activation, we measured GL3 processing in affected and control fibroblasts stimulated with SAG and quantified the ratio of GL3 full length (GL3FL) to repressor (GL3R) forms by western blot analysis. We found that this ratio is increased two- to three-fold in DYNC2LI1-mutant fibroblasts as compared with control cells (Fig. 7d,e). An increased ratio of GL3FL to GL3R suggests impaired proteolytic processing of GL3, which has been proposed to be the underlying cause for the polydactyly commonly seen in SRPS and other ciliopathies. In addition, total GL3 was markedly increased in lying cause for the polydactyly commonly seen in SRPS and other processing of GL3, which has been proposed to be the under-

Discussion

We show that DYNC2LI1 mutations cause SRPS, expanding the number of genes associated with this condition. The phenotypic findings in these SRPS cases are similar to those seen in cases with DYNC2H1 (ref. 36) and WDR34 (ref. 13) mutations, supporting that these proteins are part of the same complex. Using SRPS fibroblasts, we show that DYNC2LI1 mutations result in loss of cilia length regulation, ciliary accumulation of IFT components and aberrant Hedgehog pathway activity. Rescue of mutant fibroblasts with human DYNC2LI1 corrected the cilia phenotype, directly confirming that the changes in cilia length and IFT resulted from the loss of normal DYNC2LI1.

Our findings demonstrate that DYNC2LI1 is involved in both the repression of Hedgehog activity in the absence of stimulation and the activation of Hedgehog in response to stimulation. Alterations to Hedgehog signalling due to mutations in IFT-A proteins are typically mediated by either changes in cilia structure or trafficking, as opposed to non-ciliary functions of these proteins. In the absence of stimulation, SMO is actively excluded from the cilia and GL3 is processed into its repressor form. We show that SMO inappropriately enters the cilia in the absence of SAG stimulation in SRPS but not in control cells (Fig. 7b). This abnormal ciliary localization of SMO has been seen in other IFT mutants as well, including DYNC2H1 (ref. 41) and ICK, and is suggestive of inappropriate Hedgehog pathway activation. Whether this SMO is active or not remains to be determined; however, the deficient processing of GL3 into the repressor form suggests the former in DYNC2LI1-mutant fibroblasts (Fig. 7d,e). We observed that DYNC2LI1-mutant SRPS cells exhibit increased amounts of GL3FL with and without stimulation, increased ratios of GL3FL to GL3R and decreased amounts of GL3R upon stimulation as compared with controls. However, we could not identify a grossly abnormal transcriptional response in SRPS cells. This suggests that these cells can at least partially respond to Hedgehog signalling, though the in vivo consequences of persistent altered GL3FL to GL3R amounts in certain tissues, particularly in a temporal manner, may underlie developmental abnormalities, particularly polydactyly, as seen in these SRPS cases.

We found that mutations in DYNC2LI1 do not preclude cilia formation, but rather cause cilia length dysregulation. On the basis of the phenotype of most mutants with diminished capacities for retrograde transport, including dynein-2 complex mutants, it is interesting that cilia in DYNC2LI1 mutants are longer than those in controls. In Chlamydomonas, dynein-2 null mutants (dhc1b21; stf-1, stf-2 (ref. 22)) have short, bulbous cilia with an accumulation of IFT-B components at their tips, suggesting an essential role for dynein-2 in recycling ciliary components back to the cell body. As IFT-B accumulates in these mutants, exhausting the pool of kinesin-2, the cilium loses its ability to grow or even maintain its length. In contrast, the DYNC2LI1-mutant SRPS cases reported here have pronounced losses of both DYNC2H1 and DYNC2LI1 yet display robust ciliogenesis with longer-than-average cilia, despite having the characteristic IFT-B accumulations. This is similar to dynein-2 mutants in Tetrahymena (KO-D2LI/IC). It is thus likely that the DYNC2LI1 mutations alter the functioning of the dynein-2 complex without abolishing function altogether. The main defect in these cells appears to be loss of cilia length regulation as is evidenced by large variations in cilia lengths. It is possible that the small amount of remaining normal DYNC2H1 is enough to...
prevent the IFT congestion and stunted cilia seen in other mutants\textsuperscript{46}. We therefore conclude that the residual activity of the dynein-2 complex in \textit{DYNC2LI1}-mutant fibroblasts was enough to support robust ciliogenesis but insufficient to ensure the correct regulation of primary cilium size.

While we have shown that mutations in \textit{DYNC2LI1} produce a severe skeletal dysplasia, little is known regarding the expression of this gene in distinct tissues. qRT\textendash{}PCR from RNA derived from a series of 16-week fetal tissues showed that \textit{DYNC2LI1} is expressed in all tissues studied, but is highest in bone (Supplementary Fig. 3). Furthermore, the dynein-2 complex proteins we evaluated localized to the perichondrium/periosteum and primary spongiosa of bone (Fig. 4), suggesting an important role in the developing skeleton. These regions have been shown to be important for signalling and organization of a normal cartilage growth plate\textsuperscript{47,48}. The primary spongiosa, in particular, was reported to be irregular in SRPS cases with \textit{DYNC2H1} mutations\textsuperscript{4} with loss of degradation of the mineralized hypertrophic zone, supporting the potential role of the dynein-2 complex in orchestrating the development of normal endochondral bone formation. Localization of the dynein-2 complex to the cartilage growth plate begins to provide insight into the reasons for altered endochondral ossification that are seen in SRPS.

**Figure 5** | Primary cilia are variable in length and longer in \textit{DYNC2LI1}-mutant cells. (a) Immunofluorescence micrographs of serum-starved control and SRPS fibroblasts stained for ARL13B (green), GluTUB (red) and Hoescht (blue) show variable length and hyperelongated cilia in SRPS cells. (b) Percentage of ciliated cells in (a) (\(n = 150 \times 3\) independent experiments). (c) Primary cilia length is highly variable in cells with \textit{DYNC2LI1} mutations (\(n > 20 \times 3\) independent experiments). (d) Expression of untagged wild-type \textit{DYNC2LI1} through an IRES-GFP vector (artificially coloured blue) rescues cilia length variability in SRPS cells. Error bars represent ± s.e.m. Statistical analyses performed using Mann–Whitney test, *\(P < 0.05\). Scale bar, 5 \(\mu m\).
In summary, our findings establish the clinical and cellular consequences of DYNC2LI1 mutations and dynein-2 complex disruption in human primary cells, and support the profound importance of this protein complex in proper skeletogenesis.

Methods

Exome sequencing. Under an approved University of California at Los Angeles human subjects protocol, DNA was isolated and submitted to the University of Washington Center for Mendelian Genomics for library preparation and exome sequencing. The samples were barcoded, captured using the NimbleGen SeqCap EZ Exome Library v2.0 probe library targeting 36.5 Mb of genome, and sequenced on the Illumina GAIIx platform with 50 bp reads. Novoalign was used to align the sequencing data to the human reference genome (NCBI build 37) and the Genome Analysis Toolkit49 was used for post processing and variant calling according to Genome Analysis Toolkit Best Practices recommendations50,51. Average coverage of targeted bases was 103, 61 and 56 for R01-013A, R07-627A and R03-303A, respectively. For each sample, at least 90% of targeted bases were covered by at least 10 independent reads. Variants were filtered against dbSNP137, 95 NIEHS EGP exome samples (v.0.0.8), 6,303 exomes from the NHLBI Exome Sequencing Project (ESP6500), 1,000 genomes (release 3.20120430) and 40 in-house exome samples. Mutations were further compared with known disease-causing mutations in HGMD (2012.2). Variants were annotated using VAX52 and mutation pathogenicity was predicted using the programs Polyphen53, Sift54, Condel55 and CADD56. The mutations reported in this work were confirmed by bidirectional Sanger sequencing of amplified DNA from the proband and available parents (Supplementary Fig. 1). Primer sequences used were:

DYNC2LI1-ex6 F: 5'-TTTTGTGTTTGGAGATCTTAGAA-3'  
R: 5'-TTGCTGTAATTTCGCCAGTGAACA-3'

DYNC2LI1-ex12 F: 5'-ACCTGGGAGAATTGGAAGTG-3'  
R: 5'-GGTGGAACAGAGGATTT-3'

DYNC2LI1-ex13 F: 5'-GTGCTGCTTAGCCACACAGA-3'  
R: 5'-GTGGGCTTTAACCTTGCAT-3'

Sequence trace files were aligned and analysed using Geneious version 7.1.4 created by Biomatters (http://www.geneious.com/).

The samples were part of a larger cohort of SRPS samples submitted for the purpose of identifying novel disease genes and characterizing the incidence of mutations in known genes. The cohort had not been previously tested for mutations in known SRPS-causing genes.

Filtering strategy. We assigned priority to mutations predicted to affect protein function (insertions or deletions, frameshifts, non-synonymous mutations and essential splice site mutations). We later included highly conserved splice region variants, allowing us to discover the DYNC2LI1 +3 splice site mutation. We assigned priority to mutations that had not been previously seen in the homozygous state in control databases and mutations with a minor allele frequency <0.005. Due to the recessive inheritance of SRPS, we looked for genes in which...
Figure 7 | Disruption of the dynein-2 complex leads to aberrant Hedgehog signalling. (a) Immunofluorescence micrographs of control and SRPS cells treated with SAG and stained for GLI3 (red), ActTUB (green) and 4,6-diamidino-2-phenylindole (blue). (b) DYN2LI1/dynein-2 complex activity is required for the exclusion of SMO from the primary cilium in the absence of Hedgehog stimulation. Immunofluorescence micrographs of SAG-stimulated control and SRPS cells stained for SMO (green) and GluTUB (red). (c) Graph shows the mean percentage of ciliated cells with ciliary SMO ± s.e.m. (> 100 cells counted × three independent experiments). (d) Immunoblotting for GL3 (full-length ‘GLI3FL’; repressor ‘GLI3R’) in cell extracts shows increased GLI3FL to GLI3R in SRPS cells. (e) Graph shows the ratio of GLI3FL to GLI3R. (f) Graph shows quantification of total GLI3 amounts (GLI3FL + GLI3R), which is increased in DYN2LI1-mutant fibroblasts. Statistical analyses performed using Mann–Whitney test, *P < 0.05. Scale bars, 5 μm.
both alleles were potentially mutated. DYNC2LI1 stood out as a potential gene due to the incidence of unique, compound heterozygous mutations in three families in which the SRPS cases had strikingly similar X-ray features.

RT-PCR. RNA from SRPS case and control fibroblast cell lines was extracted using TRIZOL. RT–PCR was performed using SuperScript III One Step Kit (Invitrogen 12574-018) with the following DYNC2LI1 primers targeting a 431 bp template spanning exon 12:

F: 5′-CCGTTGTTTACACAGGAGGA-3′
R: 5′-CCAAGAAATACGCAGCTC-3′

An amplified fragment of 431 bp corresponds to the wild-type RNA and a fragment of 339 bp corresponds to a transcript missing exon 12.

Quantitative RT-PCR. Total RNA was extracted from a series of 16-week fetal tissues as well as from control, R01-013A and R07-628A fibroblasts (with and without SAG treatment) using TRIZOL (Invitrogen 15596). cDNA was treated with DNase (Invitrogen 18068) and reverse transcribed (Thermo Scientific K1621) according to the manufacturer’s instructions. qRT–PCR primers sequences were as follows: DYNC2LI1 F: 5′-GAAGGTGTAGGCTGAACTTTG-3′; R: 5′-GTATATCTCAAGGATGTTGTT-3′

YWHAZ F: 5′-CACGAGATGCTGAGAATA-3′; R: 5′-GAAGCATGGGGATCAAC-3′

BACT F: 5′-TCCCTGGGAAGAGACTCAAG-3′; R: 5′-AGGAAGAAGAGCTGGGAAGAG-3′

CTTGAAGAG-3′

PTCH1 F: 5′-ACCTCTGGTGGAGCTGTGAT-3′; R: 5′-GGAAUAAUAUGCCGAAGGA-3′

GLI3 F: 5′-ACAGCCGTACACCTGGTCC-3′; R: 5′-GGCTGACAGTATAGCGGCTGAC-3′

BAPDH F: 5′-GTAGTACGTCGCGCCTACAGG-3′; R: 5′-GCTGGGCGGCTCTAGA-3′

GUSB F: 5′-GATCAAGAAATACGCAGCTC-3′; R: 5′-CCCTGGGAGAGCTCAGCTA-3′

Molecular cloning of DYNC2LI1. Human DYNC2LI1 cDNA (NM_016008.3) was synthesized using gBlock from IDT. The cDNA was flanked by Xhol and NotI restriction sites, which were used for insertion into pCAGG, a pCAG-IREs vector (a gift from Connie Cepko (Addgene plasmid #11159)). The final construct synthesized using gBlock from IDT. RT–PCR was performed with a real-time PCR detection system (Stratagene MX3005P) using SYBR Green PCR master mix (Stratagene). Standard thermocycler conditions, qRT–PCR primer pairs were performed in triplicate in a total volume of 25 μl for 3 independent replicates. Each sample was analysed for two housekeeping genes to normalize for RNA input amounts and to perform relative quantifications. Levels of transcripts in controls were set at 1. Melting curve analysis showed a single, sharp peak with the expected melting temperature for all samples.

For serum starvation and to promote ciliogenesis, cells were washed in PBS and incubated without serum for 24 h. For induction of the sonic hedgehog pathway, serum-free media was supplemented with 20 ng/ml of SAG. For knockdown, cells were transfected with siRNA smartpool (Dharmacon) against the following target sequences:

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Author contributions

S.P.T., D.C and D.K. conceived of the study; S.P.T., T.D. and D.K. wrote the manuscript; R.L., D.C. and D.K. defined radiographic features and identified the SRPS phenotype; S.P.T., T.D., D.K. and R.V. designed and supervised experiments and were involved in data interpretation; U.W.C.M.G. and S.F.N. provided whole-exome sequencing data and S.P.T. and S.W. performed whole-exome sequencing analysis; S.P.T. and T.D. performed Hedgehog stimulation experiments, Western blots, RT–PCR and immunofluorescence analysis of human fibroblasts; I.D. performed immunohistochemistry of human cartilage growth plate and qPCR experiments; S.P.T. performed Sanger sequencing of human samples; T.D. performed ciliary and IFT measurements, RNAi knockdown and rescue experiments on patient fibroblasts; D.K. was involved in patient enrolment; S.F.N. provided computational infrastructure for data processing.

Additional information

Accession codes: The sequence data have been deposited in ClinVar under the accession codes SCV000212251, SCV000212252, SCV000212253, SCV000212254, SCV000212255 and SCV000212256.

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