Mutations in PLK4, encoding a master regulator of centriole biogenesis, cause microcephaly, growth failure and retinopathy

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Centrioles are essential for ciliogenesis. However, mutations in centriole biogenesis genes have been reported in primary microcephaly and Seckel syndrome, disorders without the hallmark clinical features of ciliopathies. Here we identify mutations in the genes encoding PLK4 kinase, a master regulator of centriole duplication, and its substrate TUBGCP6 in individuals with microcephalic primordial dwarfism and additional congenital anomalies, including retinopathy, thereby extending the human phenotypic spectrum associated with centriole dysfunction. Furthermore, we establish that different levels of impaired PLK4 activity result in growth and cilia phenotypes, providing a mechanism by which microcephaly disorders can occur with or without ciliopathic features.

Centrioles are microtubule-based structures that form the core of the centrosome and nucleate cilia and flagella. Centrioles are therefore key components of many cellular processes, including cell division, signaling and motility1–3. Centriole number is tightly regulated during the cell cycle to ensure accurate chromosome segregation at mitosis4,5. During G1 phase, the cell possesses two centrioles, which are duplicated during the S and G2 phases. Pericentriolar material (PCM) then forms around each pair of centrioles, generating two centrosomes that separate and move to opposite ends of the cell to coordinate bipolar spindle formation and chromosome segregation.

A core set of evolutionarily conserved proteins is required for centriole biogenesis6–5. Many of these components have been implicated in human microcephalic disorders6–11. Mutations in CENPJ (CPAP), CEP152, CEP135, CEP63 and STIL have been identified in primary microcephaly, an autosomal recessive disorder of cerebral cortex size, and mutations in two of these genes (CENPJ and CEP152) can also cause microcephalic primordial dwarfism, in which extreme reduction in brain size is seen alongside prenatal and postnatal reduction in body size. No ciliopathy-associated phenotypes have been reported in any of these cases.

Here we report the identification of mutations in PLK4 and its phosphorylation target TUBGCP6 in individuals with microcephaly, primordial dwarfism and other clinical features, extending the phenotypic spectrum associated with centriole biogenesis genes.

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RESULTS
Identification of mutations in \textit{PLK4} and \textit{TUBGCP6}

We ascertained a consanguineous family with seven affected individuals with microcephalic dwarfism from the Khyber Pakhtunkhwa (KPK) province of Pakistan (Fig. 1a, Table 1 and Supplementary Table 1). Genome-wide linkage studies on this family by Illumina BeadChip SNP genotyping established a new disease-associated locus of 11.7 Mb comprising 56 genes on chromosome 4 with a statistically significant logarithm of odds (LOD) score of $z = 4.7$ (Supplementary Fig. 1a,b). Exome sequencing of one affected individual from this family identified a homozygous intronic mutation, c.2811–5C>G, of 11.7 Mb comprising 56 genes on chromosome 4 with a statistically significant logarithm of odds (LOD) score of $z = 4.7$ (Supplementary Fig. 1a,b). The variant was not present in 286 Pakistani controls or in the 1000 Genomes Project and National Heart, Lung, and Blood Institute Grand Opportunity (NHLBIGO) Exome Sequencing Project (ESP) public variant databases.

Independently, exome sequencing was also performed on a 15-year-old African case with extreme microcephaly, short stature, retinopathy and deafness (subject P6, Table 1). After filtering for common variants (minor allele frequency (MAF) > 0.005), analysis under a recessive model of inheritance identified a homozygous frameshift mutation in exon 5 of \textit{PLK4} (c.1299_1303delTAAAG; p.Phe433Leufs*6), which was confirmed by capillary sequencing in this and other affected family members. This mutation was predicted to create a new splice acceptor site in intron 15, incorporating 4 bp of intronic sequence into the transcript and causing a frameshift and premature truncation of the protein (p.Arg936Serfs*1) (Table 1). The variant was not present in 286 Pakistani controls or in the 1000 Genomes Project and National Heart, Lung, and Blood Institute Grand Opportunity (NHLBIGO) Exome Sequencing Project (ESP) public variant databases.

Capillary resequencing of the coding exons and splice-site junctions of \textit{PLK4} in 318 primary microcephaly and primordial dwarfism cases led to the identification of a second individual homozygous for the c.1299_1303delTAAAG mutation. Strikingly, the two cases were from entirely distinct geographical regions: P6 was from Madagascar and P7 was from Iran. However, microsatellite genotyping of the region surrounding \textit{PLK4} identified an ancestral haplotype shared by both cases, extending 2.9 Mb from 126.3 to 129.2 Mb on chromosome 4 (hg19) (Supplementary Fig. 1c), consistent with shared remote ancestry many generations ago.

As \textit{PLK4} is a key regulator of centriole biogenesis\cite{12,13} and given the genetic mapping data identifying distinct, independent mutations predicted to substantially disrupt gene function, we concluded that \textit{PLK4} was a newly discovered disease-related gene.

Phenotypically, the individuals with \textit{PLK4} mutations displayed profound microcephaly (head circumference, $−11.6 ± 2.7$ s.d.) and substantial growth retardation of prenatal onset, with markedly reduced height ($−5.5 ± 2.1$ s.d. at current age), in keeping with the diagnosis of microcephalic primordial dwarfism (Fig. 1a–c, Table 1 and Supplementary Table 1) on the primary microcephaly–Seckel syndrome spectrum\cite{14}. Severe intellectual disability was evident in all cases, and in some individuals there were additional neurological deficits (Supplementary Note). Neuroimaging demonstrated marked reduction in cortical size with simplified gyral folding (Fig. 1d). Cerbellar and brain stem size were also reduced, with collections of increased cerebrospinal fluid evident, particularly in P2 and P7 in whom large interhemispheric arachnoid cysts were seen (Supplementary Fig. 2).

Ocular anomalies were also frequently observed. In family 1, eye size was reduced in some affected individuals, most prominently in P4 who had the complete absence of one eye (magnetic resonance imaging (MRI); Supplementary Fig. 2c). In P6, the results from detailed ophthalmological assessment were available: fundus examination showed pale optic disks, thin retinal vessels and bilateral macular atrophy. The electroradiogram (ERG) was non-recordable, reflecting a severe generalized retinopathy. Subsequent exome sequencing of an unrelated individual with primordial dwarfism and retinopathy identified a homozygous frameshift mutation in \textit{TUBGCP6} (c.433insT; p.His1445Leufs*24). In this individual, the ERG also demonstrated the complete absence of rod and cone function. Notably, \textit{TUBGCP6} is a direct phosphorylation target of the PLK4 kinase\cite{15}, suggesting that these proteins might act in the same cellular pathway to cause the microcephaly and retinopathy phenotypes. Subsequent screening of 12 additional individuals with microcephaly and retinal dystrophy identified 3 further cases with \textit{TUBGCP6} mutations, all of whom were phenotypically similar to the

Figure 1 Individuals with mutations in PLK4 or TUBGCP6 exhibit extreme microcephaly and short stature. (a) Pedigree for family 1, a large consanguineous Pakistani family with microcephalic primordial dwarfism, and photographs of affected individuals. An asterisk indicates family members from whom DNA was available. Genotype at the mutation site (GG or GC) is indicated. (b) Photographs of additional individuals with PLK4 or TUBGCP6 mutations. (c) Head circumference is disproportionately reduced relative to height. Growth parameters are plotted as the s.d. (z score) from the population mean for individuals of matched age and sex. Circles, patients with PLK4 mutations; squares, patients with TUBGCP6 mutations. OFC, occipitofrontal circumference. (d) Axial T2-weighted MRIs showing that the cerebral cortex is substantially reduced in size with simplified gyral folding in patients. Images are shown for P1 at 18 months of age (OFC, $−14.0$ s.d.) and P6 at 20 years of age (OFC, $−9.0$ s.d.). Images from age-matched controls are shown below. Scale bar, 2 cm. Parents provided written consent for the publication of the photographs of the affected individuals.
individuals with PLK4 mutations (Table 1, Supplementary Table 1 and Supplementary Note). As with family 1, microphthalmia was also seen in family 6. Identification of these mutations substantiates a previous case report of microcephaly and chorioretinopathy in an Amish individual for whom a homozygous TUBGCP6 mutation disrupting the termination codon was detected by exome sequencing. Given the previous publication on TUBGCP6, we focused subsequent investigations on the functional consequences of mutations in PLK4 because of its central importance in centriole biogenesis and the availability of primary cell lines from individuals with PLK4 mutations.

Transcriptional and protein consequences of the PLK4 mutations

In family 1, the c.2811–5C>G mutation was predicted to create a new splice acceptor site, resulting in the addition of 4 bp of the
intron 15 sequence to the transcript and premature truncation of the PLK4 protein in the last polo-box domain (Fig. 2a,b). RT-PCR confirmed that this mutation altered splicing (Fig. 2c), with no residual wild-type transcript detectable in RNA from the affected individual (Supplementary Fig. 3a).

In families 2 and 3, the homozygous c.1299_1303delTAAAG mutation in exon 5 of PLK4 (Fig. 2a,b) resulted in a frameshift introducing a 5-bp deletion (p.Phe433Leufs*6), predicted to result in an early termination codon (p.Phe433Leufs*6). Capillary sequencing of RT-PCR products confirmed the predicted splice-site junctions of the ALT and FL transcripts (Supplementary Fig. 4b). Notably, the alternative splice site was only present in apes and was not found in other primates, as a single-nucleotide substitution at this site results in a derived allele that creates a consensus splice-site sequence (Supplementary Fig. 4c).

The identification of an alternative transcript encoding an internal 60-residue deletion that lay outside of a known functional domain suggested that the 5-bp deletion would lead to reduced PLK4 activity rather than complete loss of PLK4 function. To address this possibility, we first investigated the effect of this mutation on transcript levels by quantitative RT-PCR of PLK4 (ALT) and PLK4 (FL). As anticipated, PLK4 (FL) transcript levels were substantially reduced (Supplementary Fig. 5a), consistent with nonsense-mediated decay of the prematurely terminating transcript. In contrast, PLK4

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**Figure 3** PLK4 mutations impair PLK4 activity in centriole biogenesis, resulting in reduced centriole number in patient-derived cells. (a,b) Centriole overduplication assay demonstrating that transfected PLK4 (FL) and PLK4 (ALT) GFP-tagged constructs are competent for centriole duplication, whereas the Phe433Leufs*6 and Arg936Serfs*1 mutants have significantly reduced activity. (a) Representative images of HeLa cells transfected for 48 h with eGFP–PLK4 constructs (immunoblot in Supplementary Fig. 8) demonstrating centriole overduplication with PLK4 (FL) and PLK4 (ALT) but not with truncated constructs representing patient-identified mutations. Centrosomes are highlighted in the boxes. CENT3, centrin-3. Scale bar, 4 µm. (b) Quantification of the experiments in a. n = 3 experiments, with 100 cells/experiment. P values were calculated by two-tailed t test: **P < 0.001; ***P < 0.0001; NS, not significant. Error bars, s.e.m. (c,d) At mitosis, 6–12% of fibroblasts derived from individuals with PLK4 mutations have reduced centriole number. (e) Centrin foci quantified in prometaphase and metaphase fibroblasts (n = 3 experiments, with 200 cells/experiment). Error bars, s.e.m. P values were calculated by two-tailed t test: **P < 0.01, *P < 0.05. (d) Quantification of the centriole phenotypes observed in the 12% of mitotic P6-derived fibroblasts with reduced centriole number (fibroblasts with ≤2 centrioles (n = 3 experiments, with 175 cells/experiment)). “2 together” indicates two centrioles detected at one spindle pole; “2 separate” indicates two centrioles detected, one at each pole. (e) Mitotic spindle formation is impaired in P6-derived fibroblasts with reduced centriole number. Left, representative images. Insets of centrin-3 staining are shown at 3x magnification. Balanced, cells with a broad-based bipolar spindle; unbalanced, cells with an unequal bipolar spindle; monopolar, cells with only one spindle pole; disorganized, cells that failed to establish a spindle pole. ATUB, α-tubulin. Scale bar, 10 µm. Right, quantification of the mitotic spindle phenotypes observed in the 12% of mitotic P6-derived fibroblasts with reduced centriole number (n = 3 experiments, with 75 cells/experiment).
Figure 4 Depletion of pklk4 causes dwarfism in zebrafish. (a) Schematic of the intron-exon structure of the zebrafish pklk4 gene. Red bars indicate splice sites targeted by morpholino oligonucleotides (MOs). Arrows indicate the positions of RT-PCR primers (F, forward; R, reverse). (b) Dose-dependent depletion of transcript levels in pklk4 morphants. Transcript levels were measured by quantitative RT-PCR of RNA extracted at 2 d.p.f. Transcript levels are shown relative to those for embryos injected with control morpholino at 2 d.p.f. (n = 3 experiments; error bars, s.e.m.). (c) Depletion of pklk4 with 0.5 ng of pooled morpholinos results in small, morphologically normal ‘dwarf’ zebrafish embryos. Representative images are shown of embryos injected with either 0.5 ng of pklk4 morpholinos or 12 ng of control morpholino at 5 d.p.f. Scale bar, 1 mm. (d) Body surface area is significantly reduced in zebrafish injected with pklk4 morpholino at 5 d.p.f. Effects on size can be rescued by coinjection with zebrafish pklk4. Surface area is expressed as a z score relative to un.injected embryos of the same age (the z score is defined as the s.d. from the mean size for un injected embryos at 5 d.p.f.). P values were calculated by two-tailed t test: ***P ≤ 0.001 versus control embryos and, for RNA coinjection rescue experiments, versus embryos injected with 1.5 ng of pklk4 morpholino alone. Error bars, s.e.m. (e) Cell number is reduced in pklk4 morphants. Time course from 0.5–48 hours post-fertilization (h.p.f.) for the indicated pklk4 morpholo nin concentrations. At each time point, 50 embryos per dose in triplicate. Error bars, s.e.m. (f) Wild-type human PLK4 mRNA also partially rescues the aberrant growth phenotype, whereas mRNAs encoding truncated protein products representing the human alterations p.Phe433Leufs*6 and p.Arg936Serfs*1 do not complement the aberrant growth phenotype. n = 3 mating pairs (>50 embryos analyzed for each rescue construct in each mating pair). P value were calculated by two-tailed t test: **P ≤ 0.01, *P ≤ 0.05 versus embryos injected with 1.5 ng of pklk4 morpholino alone. NS, not significant. Error bars, s.e.m.

(ALT) was stably expressed in patient-derived cells (Supplementary Fig. 5b), such that the total amount of PLK4 transcript available to produce functional PLK4 enzyme in these cells was reduced to 25.7 ± 2.8% of control levels (Fig. 2e).

We next determined the consequences of the mutations on PLK4 transcript and protein levels in these cells. Immunoblotting was performed to characterize the effects of the mutations on the cellular levels of the PLK4 protein. Two PLK4-specific bands were evident in immunoblotting with a published antibody to PLK4 (ref. 18) (Fig. 2f), corresponding to the FL and ALT isoforms. In fibroblasts derived from P1 and P7, the slower-migrating band was absent, consistent with loss of the FL isoform from these cells. The intensity for the band in P1-derived fibroblasts was marked reduced and the band appeared to have increased mobility relative to the FL band, in keeping with reduced protein size due to the C-terminal truncation. Additionally, we measured PLK4 accumulation at the centrosome by immunofluorescence (Fig. 2g and Supplementary Figs. 6 and 7). As the immunofluorescence signal in primary fibroblasts was too weak to quantify, we treated cells with the proteasomal inhibitor MG132 before fixation to enhance PLK4 detection, permitting robust, reproducible measurements. Quantitative analysis of the immunofluorescent signal established that PLK4 levels were significantly reduced (P < 0.0001), with PLK4 protein levels 15 ± 0.02%, 36 ± 1% and 42 ± 0.08% of control levels in fibroblasts derived from P1, P6 and P7, respectively (Fig. 2g).

We concluded that both mutations result in reduced levels of PLK4 protein in the cell. For the p.Phe433Leufs*6 alteration (P6 and P7), this occurs as the consequence of nonsense-mediated decay of the FL transcript and consequent loss of the FL protein. For the p.Arg936Serfs*1 alteration (P1), the frameshift occurs in the terminal exon, so nonsense-mediated decay would not be expected. However, structural modeling of the terminal polo-box domain (PB3) indicated that this domain would be substantially disrupted in the mutant protein (Supplementary Fig. 3b). Therefore, the marked reduction in protein levels in these cells is likely due to a consequent reduction in protein stability.

PLK4 mutations impair centriole biogenesis

We assessed the functional activity of both full-length isoforms and mutant PLK4 proteins using an overexpression assay. Enzyme activity, determined by the overduplication of centriole number, was significantly impaired for both mutant proteins, indicating that such residual mutant protein would also be functionally impaired (Fig. 3a, b and Supplementary Fig. 8). In contrast, the PLK4 (ALT) protein was as active as PLK4 (FL), consistent with a similar functionality in centriole duplication.

Given the consequences of the mutations on PLK4 transcript and protein, centriole duplication would be predicted to be impaired but not absent in cells derived from affected individuals. As centriole number changes over the course of the cell cycle, mitosis provides a convenient stage to quantify centriole number, with four centrioles normally present at this time. In keeping with their reduced PLK4 function, patient-derived fibroblasts had a statistically significant reduction in centriole number, with the number of prometaphase
Figure 5 Impaired mitosis leads to growth retardation in plk4-morphant zebrafish. (a) Centriole number is reduced in mitotic cells from plk4 morphants. Left, representative images of mitotic cells from embryos injected with control or plk4 morpholino at 2 d.p.f. CENT3, centrin-3; pH3, phosphorylated histone H3 (marker of mitotic chromatin). Insets of centrin-3 staining are shown at 3× magnification. Scale bar, 2.5 µm. Right, quantification of centrin-3 foci in zebrafish cells isolated from zebrfish at 2 d.p.f. injected with control morpholino or 0.5 ng of plk4 morpholino (n = 2 experiments, with >100 mitoses/experiment; error bars, s.e.m.). (b) Increased numbers of mitotic cells are present in zebrafish injected with 0.5 ng of plk4 morpholino at 2 d.p.f. Left, representative FACS plot of DNA content versus phosphorylated histone H3 staining of a single-cell suspension from embryos at 2 d.p.f. Mitotic cells have 4N content and are positive for phosphorylated histone H3 (blue). Cells in sub-G1 phase are shown in black. Right, quantification of 3 experiments (n = 50 embryos/experiment; *P < 0.05; error bars, s.e.m.). (c) Aberrant mitotic spindles are frequently seen in plk4-morphant embryos at 2 d.p.f. Left, insets of centrin-3 staining are shown at 3x magnification. ATUB, α-tubulin. Scale bar, 5 µm. Right, quantification of the mitotic phenotypes observed in plk4-morphant embryos with reduced centriole number (n = 100 cells, 1 experiment).

and metaphase cells containing fewer centrioles (two or fewer centrin foci) significantly higher in comparison to two control lines (P1, 12% of cells (t test, P = 0.04); P6, 10.2% of cells (t test, P = 0.0004); P7, 5.8% of cells (P = 0.04); Fig. 3c). Further characterization of the centriolar phenotype in mitotic cells from P6 established that cells with reduced centriole number predominantly had a single centriole pair (60%) or were cells with no centriole (25%) (Fig. 3d).

As centrioles are key centrosomal components, centriolar depletion would be expected to impair mitotic spindle formation. Accordingly, normal mitotic spindle formation was affected in patient-derived cells with reduced centriole number, with monopolar spindles most frequently observed (Fig. 3e). Delayed mitotic progression would therefore be predicted in these cells, as a balanced bipolar spindle is required for efficient mitosis.20 At least some of these cells eventually progressed through mitosis, as anaphase cells with a single centriole pair were observed (data not shown) and an increase in the number of interphase cells without centrioles was seen (P6, 3.9%, P = 0.03; P7, 2.5%, P = 0.01; Supplementary Fig. 9). Abnormalities in the mitotic spindle can lead to mitotic errors, including chromosome segregation defects, cytokinesis failure or cell death. However, no significant increase in the number of cells with >4N DNA content was observed by FACS analysis (data not shown), nor was increased cell death detected by Annexin V staining (Supplementary Fig. 10). Additionally, chromosome segregation errors during anaphase were rarely observed (Supplementary Table 2), and interphase FISH also did not demonstrate a significant increase in aneuploidy or tetraploidy (Supplementary Fig. 11).

We therefore concluded that reduced PLK4 activity results in impaired centriolar duplication and leads to impaired mitotic spindle formation in a subset of cells. To address the developmental consequences of such cellular PLK4 dysfunction, we next established a zebrafish model.

**plk4 depletion impairs mitosis and reduces zebrafish size**

As our cellular studies established that mutations reduced PLK4 levels, we used morpholino antisense oligonucleotides to deplete plk4 transcripts in zebrafish, a system previously used to model primordial dwarfism due to mutations in ORC1 (ref. 21). We designed splice site–blocking morpholinos to target the splice donor and acceptor sites of exon 5 of zebrafish plk4 (Fig. 4a). Titration of pooled morpholino oligonucleotides injected into embryos at the one-cell stage permitted depletion of plk4 transcript to 2–25% of control levels in embryos at 1 day post-fertilization (d.p.f.) (Fig. 4b), with 0.5 ng of plk4 morpholino achieving transcript levels comparable to those seen in fibroblasts derived from the individuals with PLK4 mutations (Fig. 2e). Overall body size was significantly reduced in plk4 morphants at 5 d.p.f. (Fig. 4c,d and Supplementary Fig. 12), with reduction in size correlating with the extent of transcript depletion (Fig. 4d). Reduction in embryo size was a consequence of reduced cell number (Fig. 4e), with cell size remaining unchanged (Supplementary Fig. 13). Such reduction in cell number was evident from early on in development (Fig. 4e) and could be the consequence of reduced cell proliferation or increased cell death. Reduced growth was a direct effect of plk4 depletion rather than morpholino-induced toxicity, as growth was rescued by coinjection of embryos with 150 pg of zebrafish plk4 mRNA and 1.5 ng of plk4 morpholino (P < 0.05; Fig. 4d). When human PLK4 (FL) and PLK4 (ALT) mRNAs were used for coinjection, both partially rescued the dwarfism phenotype, indicating that both isoforms can functionally contribute to organism growth (Fig. 4f), whereas PLK4 mRNAs encoding the Arg936fs*1 and Phe433Leufs*6 mutants both failed to rescue the aberrant growth phenotype.

To gain insight into the mechanism underlying reduced growth and cell number, we disaggregated plk4 morpholino–injected zebrafish embryos and examined embryonic cells by immunofluorescence and FACS analysis. Immunostaining for centrin-3 established that reduced plk4 levels also impaired centriolar biogenesis during development,
with the majority of mitotic cells having fewer than the expected four centrioles (65% of mitotic cells in plk4 morphants versus 7% in control embryos; P = 0.0004; Fig. 5a). We postulated that cell cycle progression would consequently be impaired, so we next performed FACs cell cycle analysis, which demonstrated a significant accumulation of cells positive for phosphorylated histone H3 in plk4 morphants (3.9% of cells versus <0.56% in controls; P < 0.05; Fig. 5b). Given the reduced cell proliferation in plk4 morphants (Fig. 4e), this finding indicates a substantial delay in mitotic progression, also seen in stil-mutant zebrafish22, which likely occurs as a consequence of aberrant mitotic spindle formation (Fig. 5c) resulting from impaired centriole duplication. Spindle formation might additionally be compromised because of the role of Plk4 in acentriolar spindle formation23.

An increased rate of apoptosis has been observed for other centriole biogenesis mutants22,24–26, and we therefore also performed TUNEL staining (Supplementary Fig. 14). A low level of apoptosis was seen in zebrafish injected with 0.5 ng of plk4 morpholino, and the level increased at higher doses of morpholino, suggesting that cell death also contributes to the decrease in cell number in these embryos, although TUNEL-positive cells were often localized to the tail region and did not precisely correlate with the broad increase in the number of cells with positive staining for phosphorylated histone H3. Furthermore, unlike the parallel rescue seen in stil-mutant zebrafish with tp53 depletion22, no rescue in growth was seen in tp53-mutant zebrafish injected with plk4 morpholino (Supplementary Fig. 15).

**Photoreceptor and ciliopathy phenotypes in plk4 morphants**

Retinopathy occurs in individuals with mutations in either PLK4 or TUBGCP6 (Table 1), and, therefore, we next used our plk4-morphant model to investigate the basis of this phenotype. We found that plk4-morphant zebrafish had impaired responses to visual stimuli, with 60% having impaired light-dark adaption, even at low (0.5 ng) levels of morpholino (Fig. 6a). Immunohistological examination of the eyes demonstrated loss of photoreceptors, using antibody to zpr-1 as a marker27, as well as a variable reduction in eye size (Fig. 6b), consistent with the ophthalmological findings observed in humans with PLK4 and TUBGCP6 mutations. As cilia are key structures in the photoreceptors required for outer segment structure and function28, we performed immunostaining of cilia axonemes and basal bodies (Fig. 6c and Supplementary Fig. 16). This analysis demonstrated reduced numbers of cells containing cilia in the photoreceptor layer, with an absence of basal bodies correlating with the absence of cilia. Cilia loss was a direct effect of plk4 morpholino rather than morpholino-induced toxicity, as it could be rescued by coinjection of embryos with PLK4 mRNA (Fig. 6c).

Additional cilia-related phenotypes were observed upon injection with higher doses of plk4 morpholinos, as previously observed with cep152-depleted zebrafish29. Notably, these phenotypes were separable from the growth phenotype in a dose-dependent manner, with injections of 0.5 ng of plk4 morpholino resulting in small but structurally normal fish, whereas, at 1.5 ng and 3 ng, embryos with ciliopathy-associated phenotypes were generated, including hydrocephalus, renal cysts and ventral curvature (Fig. 7a–d). Furthermore, left-right asymmetry defects were detectable upon injecting plk4 morpholino into a transgenic reporter line Tg(bre-aavmlp:egfp) that labels heart musculature (Fig. 7c), and quantification of motile cilia in Kupffer’s vesicle demonstrated a significant decrease in the proportion of cells with cilia in embryos injected with 3 ng of plk4 morpholino (Fig. 7d). Likewise, the number of ciliated cells was also decreased in serum-starved fibroblasts derived from affected individuals (Supplementary Fig. 17). As many cells in embryos injected with plk4 morpholino had no remaining centrioles (25% had no centrioles, 38% had one centriole and 30% had two centrioles) and cilia loss correlated with the absence of basal bodies in cells derived from affected individuals, we concluded that the cilia phenotype was likely directly attributable to the absence of basal bodies.
Figure 7 The growth failure and ciliopathy phenotypes are separable in a dose-dependent manner. (a-d) Morphological ciliopathy phenotypes are evident at higher levels of plk4 depletion. (a) Embryos injected with 1.5 ng of plk4 morpholino at 5 d.p.f. exhibit ciliopathy phenotypes, including diluted brain ventricles (arrows), pronephric duct cysts (arrowhead) and ventral body axis curvature. (b) Penetration of the ciliopathy phenotypes (scored by the presence of ventral curvature) in plk4-morphant embryos injected with 0.5–3 ng of morpholino at 2 d.p.f. n = 100 embryos per dose in triplicate. (c) Heart laterality defects are observed at 3 d.p.f. in Tg(-aamip:egfp) zebrafish embryos injected with 3 ng of plk4 morpholino, with loss of asymmetry (midline) or inversion of ventricle-atrium asymmetry (reversed) (A, atrium; V, ventricle). A, anterior; P, posterior. Right, quantification of the heart laterality defects (n = 2 experiments, with >40 embryos/experiment). Error bars, s.e.m. (d) The proportion of ciliated cells is reduced in the Kupffer’s vesicles of embryos injected with 3 ng of plk4 morpholino Left, representative images from control embryos and those injected with 3 ng of plk4 morpholino at 16 h.p.f. Green, atypical protein kinase C (aPKC; vesicle marker); red, acetylated tubulin (cilium). Scale bars, 10 µm. Right, quantification of ciliated cells in the Kupffer’s vesicle (* P < 0.05; error bars, s.e.m.). (e,f) Model of disease pathogenicity. (e) Autoregulation of PLK4 results in a narrow window in which mutations impair enzymatic activity without resulting in embryonic lethality. At 50% transcript levels, protein levels are normal. Further depletion of PLK4 transcripts, as seen in individuals with PLK4 mutations, leads to protein loss and growth defects. Additional loss of cellular PLK4 activity results in cilia-related phenotypes. (f) Centriole duplication becomes inefficient at reduced PLK4 levels, with reduced centriole number impairing mitotic spindle formation and cell cycle progression. With severe reduction in PLK4 activity, cells completely lacking centrioles are generated, which are unable to form cilia, leading to ciliopathy phenotypes. Purple boxes represent centrioles, and red lines represent primary cilia.

**DISCUSSION**

Here we identify mutations in PLK4 and TUBGCP6 in microcephalic dwarfism and using cellular and developmental systems establish that, through reduction in centriole number, PLK4 mutations result in aberrant growth and retinal phenotypes.

The microcephaly and short stature seen in individuals with mutations in PLK4 and TUBGCP6 are comparable to the characteristics seen with previously reported mutations in centriolar biogenesis genes. However, additional developmental anomalies are also apparent, most prominently, a generalized retinopathy that has not been reported with other genes involved in primary microcephaly and Seckel syndrome. Although retinopathy is a consistent feature in individuals with mutations, it remains to be established whether it is an invariant feature in individuals with PLK4 mutations, as the geographical remoteness of families 1 and 3 precluded detailed ophthalmological assessment and electroretinography. Mutations in the mitotic spindle–binding motor kinesin gene KIF11 also cause microcephaly and retinopathy, but this phenotype is dominantly inherited, manifesting as chorioretinopathy and retinal folds, seemingly distinct from the retinopathy reported here. TUBGCP6 is a direct phosphorylation target of PLK4 and so might define a new pathway with specialized function in photoreceptors. Alternatively, the retinal pathology might result from perturbed mitosis in the eye or impaired cilia formation, given the loss of photoreceptor cilia in the plk4-morphant zebrafish model.

Although the biology of PLK4 has been intensively studied in recent years, the early embryonic lethality of the Plk4−/− mouse has limited developmental studies in mammals. Plk4−/− mice have not been reported to have microcephaly or reduced body size but were found to exhibit increased rates of liver and lung cancer. Although increased genome instability was thought to be due to cellular centrosome amplification and increased levels of tetraploidy, more recent studies on the same Plk4−/− embryonic fibroblasts did not demonstrate centrosome amplification or aneuploidy. Additionally, centrosomal PLK4 protein levels were normal, despite a 50% decrease in transcript levels in Plk4−/− cells, explained by the ability of PLK4 to regulate its own stability through autophosphorylation of a phosphodegron. In contrast, in cells derived from individuals with PLK4 mutations, a...
more substantial decrease (75%) in transcript levels impairs protein function, resulting in reduced centriole duplication. Therefore, human PLK4 mutations appear to result in protein levels that fall into a narrow window where autoregulation fails to fully compensate but the protein retains sufficient enzyme activity to prevent lethality (Fig. 7e).

A single pair of centrioles at one spindle pole is most frequently seen in mitotic cells derived from individuals with PLK4 mutations, suggesting that, when centriole duplication fails, the centrosome remains a highly stable structure. Acting as a single microtubular organizing center, this centriole generates a monopolar spindle12, delaying mitotic progression. Such a configuration might be more challenging for mitosis even than acentriolar spindle assembly; however, such mitoses can successfully resolve, as cells derived from affected individuals are seen without any centrioles (Supplementary Fig. 9). This resolution of mitosis likely occurs through the eventual formation of a bipolar spindle to permit chromosome segregation34. Although only a proportion of mitoses are affected (Fig. 3), during development, most cells from affected individuals would be expected to encounter monopolar spindle formation at some point over successive rounds of cell division. Such a reduction in mitotic and cell cycle efficiency could consequently be sufficient to cause substantial dwarfism over the course of cell division. Such a reduction in mitotic and cell cycle efficiency might explain why cilia and growth defects have not been reported, as mutations in other genes involved in centriolar biogenesis partially rescues PLK4 activity in the face of an intragenic frameshift deletion rescues this disparity, at least for this gene. It will be of interest to determine how centriole duplication is maintained with null mutations in other genes involved in centriolar biogenesis.

In summary, we report that mutations in PLK4 and TUBGCP6 cause microcephalic primordial dwarfism and a generalized retinopathy, extending the phenotype spectrum associated with centriole biogenesis genes, identifying a new biological pathway for retinal dysfunction and establishing that PLK4 activity is differentially required for growth and cilia-dependent processes during development.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.N., H.T., J.A., M.S.H., A.B., K.M., M.E. Hurles, J.E.M. and L.S.B. performed exome sequencing and analysis. L.S.B., C.-A.M., J.E.M., M.R.T., I.A., M.S.H. and G.N. performed sequencing, genotyping, linkage analysis and other molecular genetics experiments. C.-A.M., A.L., C.K., M.E. Harley, L.A., M.S.H., R.M., A.A.N. and I.H. designed and performed the zebrafish experiments. A. Klingseisen, L.S.B. and G.N. designed and performed the cell biology experiments. A. Klingseisen, A.L., C.K., M.E. Harley, I.A., M.S.H., R.M., A.A.N. and J.E.M. ascertainment subjects, obtained samples and/or assisted with phenotypic analysis and clinical studies. C.-A.M. and A.P.J. wrote the manuscript with help from P.N., A. Klingseisen and L.S.B. The study was planned and supervised by P.N. and A.P.J.
COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Online Methods

Research Subjects. Genomic DNA from the affected children and family members was extracted from peripheral blood using standard methods or saliva samples using Oragene collection kits according to the manufacturer’s instructions. Informed consent was obtained from all participating families, and the studies were approved by the ethics review board at the National Institute for Biotechnology and Genetic Engineering in Faisalabad, Pakistan, and the Scottish Multicentre Research Ethics Committee (04-MRE001/19). Parents provided written consent for the publication of photographs of the affected individuals.

Exome Capture and Sequencing. Whole-exome capture and sequencing was performed at CGC and at the Wellcome Trust Sanger Institute (WTSI). At WTSI, DNA was sheared to 150-bp fragments by sonication (Covaris) before whole-exome capture and amplification using the SureSelect Human All Exon 50Mb kit (Agilent Technologies). Fragments were sequenced using an Illumina HiSeq 2500 sequencing instrument. We aligned the 75-bp paired-end reads to the Genome Reference Consortium human Build 37 reference sequence using the Burrows-Wheeler Aligner (BWA). Single-nucleotide variants were called using GATK and SAMtools, and indels were called using Dindel and SAMtools. Variants were annotated with functional consequence using the Ensembl Variant Effect Predictor (VEP). Allele frequencies from the 1000 Genomes Project database were used to filter out population variants. Data sets were analyzed under a model of autosomal recessive inheritance. At CGC, DNA (1 µg) was fragmented using sonication technology (Covaris). Fragments were enriched using the SeqCap EZ Human Exome Library v2.0 kit (Roche NimbleGen) and subsequently sequenced on an Illumina HiSeq 2000 sequencing instrument using a paired-end 2 × 100-bp protocol. This sequencing generated 1.6 Gb of mapped sequences with a mean coverage of 89-fold, a 30× coverage of 87% of the target sequences and a 10× coverage of 97% of the target sequences. For data analysis, the Varband pipeline v.2.3 and filter interface was used. Primary data were filtered according to signal purity by Illumina Real-Time Analysis (RTA) software v1.8. Subsequently, the reads were mapped to the human genome reference build hg19 using the BWA v3.5 alignment algorithm. GATK (v1.6) was used to mark duplicated reads, perform local realignment around short insertions and deletions, recalibrate the base quality scores, and call SNPs and short indels.

Scripts developed in house at CGC were applied to detect protein changes, affected donor and acceptor splice sites, and overlaps with known variants. Acceptor and donor splice-site mutations were analyzed with a maximum-entropy model and filtered for changes in effect. In particular, we filtered for high-coverage (coverage > 15, quality = 25), rare (MAF < 0.005) homozygous variants (dbSNP Build 135, 1000 Genomes Project database build 20110521) and the public Exome Variant Server, NHLBI ESP, Seattle, build ESP6500. We also filtered against an in-house database containing variants from 511 exomes for individuals with epilepsy to exclude pipeline-related artifacts (MAF < 0.004). We validated the variants of the highest priority by conventional Sanger sequencing.

Capillary Sequencing. Primers were designed to amplify all exons and exon-intron boundaries of PLK4 and TUBGCP6 (Supplementary Table 3a). Genomic DNA from cases and parents (when available) was amplified, and PCR products underwent capillary sequenced on an ABI 3730 capillary sequencer. Sequence traces were analyzed using Mutation Surveyor software (SoftGenetics).

SNP and microsatellite genotyping. Eight individuals of family 1 from Pakistan were genotyped on an Illumina HumanCoreExome-12 v1.1 BeadChip (Supplementary Fig. 1). IV–1 (MC686–5), IV–16 (MC686–7), IV–17 (MC686–8), V–1 (MC686–13), V–2 (MC686–14), V–3 (MC686–18), VI–2 (MC686–11) and VI–1 (MC686–12). Genome-wide linkage analysis of the family was performed with 24,209 selected SNP markers. LOD scores were calculated with ALLEGRO. Data handling, evaluation and statistical analysis were performed as described previously.

The microsatellites present in a 14.2-Mb region flanking PLK4 were identified from public sequence databases, amplified from genomic DNA by PCR using fluorescently labeled primers (primer sequences are listed in Supplementary Table 3b) and genotyped on an ABI 3730 capillary sequencer.

Plasmid Constructs. The zebrafish plk4 expression construct was generated by PCR amplification of full-length plk4 from the CDNA of zebrafish embryos at 5 h.p. and TA cloning of the resulting fragment into the pGEMEase vector (Promega). Full-length human PLK4 was generated by PCR amplification of PLK4 from the IRATp970C1132D plasmid (Source Bioscience). PLK4 c.2811–5C>G, PLK4 c.1299_1303delTAAAGG and PLK4 (ALT) were generated from the full-length clone using PCR amplification. The PCR fragments were then shuttled into pDEST-EGFP (LMBP, 4542) and pCDNA3.1-MYCHIS-DEST using Gateway cloning (Life Technologies). Capped plk4 mRNA for injection was transcribed in vitro from linearized DNA using the MessageMachine Transcription kit (Ambion) and purified using the MegaClear transcription cleanup kit (Ambion).

RT-PCR. RNA was extracted from human primary fibroblast cell lines, an African green monkey fibroblast cell line, the Madin-Darby canine kidney (MDCK) cell line, the Chinese hamster ovary (CHO) cell line, a mouse embryonic fibroblast (MEF) cell line, a chicken B-lymphocyte cell line and zebrafish embryos. Human fetal retina RNA (AmsBio, R1244108–10), total bovine brain RNA (AmsBio, RB34035), total guinea pig RNA (AmsBio, GR-201), FirstChoice Human Total RNA Survey Panel (Ambion) and Marathon Ready adult retina cDNA (Clontech, 639349) were also purchased as a source of tissue.

Total RNA was isolated using the RNeasy kit (Qiagen) or TRIzol (Life Technologies) according to the manufacturer’s instructions. DNA was removed by treatment with DNAase I (Qiagen), and cDNA was generated using random oligonucleotide primers and AMV RT (Roche). The RT-PCR primer pairs used in this study are listed in Supplementary Table 3c.

Quantitative RT-PCR for human and zebrafish transcripts was performed using Brilliant II SYBR Green qPCR Master Mix (Stratagene) on the ABI Prism 7900 Sequence Detection System (Applied Biosciences). The relative expression of target genes in comparison to a control was calculated using the comparative Ct method (2−ΔΔCt) and represented as the fold induction in mRNA levels. The quantitative PCR primers used are listed in Supplementary Table 3d.

Cell culture. Dermal fibroblasts were obtained by skin punch biopsy and were cultured in amnioMAX C-100 complete medium (Life Technologies) and maintained in a 37 °C incubator with 5% CO2 and 3% O2. HeLa cells were obtained from the European Collection of Cell Cultures (93021013) and cultured in DMEM (Life Technologies), 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. hiTERT RPE-1 cells were obtained from the American Type Culture Collection (CRL-4000) and cultured in DMEM:F12, 10% FCS, 0.26% sodium bicarbonate, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were routinely tested for mycoplasma. To induce ciliogenesis, cells were incubated in low-serum (0.5% FCS) DMEM for 48 h.

Short interfering RNA (siRNA) oligonucleotides were transfected into primary fibroblasts using Dharmfect 1 (Thermo Fisher) according to the manufacture’s instructions. The oligonucleotide sequences used are listed in Supplementary Table 3e. PLK4 expression vectors were transfected into HeLa cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Where indicated, cells were treated with 10 µM MG132 (Cayman Chemicals) or 1.5 µM staurosporine (Alexis Biochemicals) for 5 h.

Interphase FISH. Nuclear suspensions were generated using standard methods from patient-derived fibroblasts incubated in hypotonic buffer (0.25% KCl) before fixation with 3:1 methanol:acetic acid. FISH was performed using centromere chromosome enumeration (CEP) probes for chromosomes 3, 4, 7, 10, 17 and 18 (Abbot Molecular) labeled in Spectrum Orange, Spectrum Green or Spectrum Aqua in CEP hybridization buffer. Slides were scanned on the OLS-120 Automated Slide Scanner at 60× magnification and analyzed using Cytovision version 7.3.1 (Leica Biosystems).

Immunofluorescence and microscopy. Primary fibroblasts were grown on untreated coverslips. Zebrafish embryo cells were adhered to coverslips coated with poly-L-lysine (Becton Dickinson). Cells were either fixed in methanol (−20 °C) for 7 min or 4% paraformaldehyde (TAAB) in PHEM (25 mM HEPES-NaOH, pH 6.8, 100 mM EGTA, 60 mM PIPES, 2 mM MgCl2) for 15 min. After fixation with paraformaldehyde, cells were permeabilized by
treatment in 0.2% Triton X-100 in PHEM for 2 min. Fixed cells were blocked in 1% BSA (Sigma-Aldrich) in PBS. Primary antibodies used were to γ-tubulin (Sigma, T5192; 1:500 dilution), α-tubulin (Sigma, T6074; 1:1,000 dilution), PLK4 (ref. 18; 1:100 dilution), centrin-2 (Santa Cruz Biotechnology, sc-27793; 1:000 dilution), centrin-3 (Novus Biologicals, H0001070-M01; 1:000 dilution), histone H3 phosphorylated at Ser10 (Cell Signaling Technology, 9701; 1:1,000 dilution), glutamylated tubulin50 (1:1,000 dilution), adenylate cyclase III (Santa Cruz Biotechnology, sc-32113; 1:100 dilution), IFT88 (Proteintech, 13967-1-AP; 1:1,000 dilution) and IFT140 (Proteintech, 17460-1-AP; 1:250 dilution). Secondary antibodies were used Alexa Fluor 488–conjugated antibody to mouse IgG (Life Technologies, A11029; 1:500 dilution) and Alexa Fluor 568–conjugated antibody to rabbit IgG (Life Technologies, A11036; 1:500 dilution). Imaging was performed using an Axioplan 2 wide-field fluorescence microscope (Zeiss) controlling a CoolSnap camera (Roper Scientific). Images were captured using a 100× Plan-APOCHROMAT (1.4 NA) objective with 0.2-μm z sections and subsequently deconvolved using Velocity software (PerkinElmer). For centrosome scoring, when three centrin signals were seen, these were binned with the four centrin signal counts, as normal centrosomal number can sometimes only be resolved into three signals by conventional light microscopy because of limited spatial resolution51,52. Fluorescence intensity analysis of three-dimensional data sets was performed using Velocity. A region covering each centrosome was defined in a three-dimensional data set, and the signal over all the sections was summed. The intensity values for a cytoplasmic background of matched volume were obtained and subtracted from the centrosome signal.

**Protein blotting.** Cells were lysed in 50 mM Tris-HCl, pH 8, 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 0.2 mM EGTA and 10% glycerol supplemented with a protease inhibitor tablet (Roche). Protein samples were run on a 6–12% acrylamide gel, and immunoblotting was performed using antibodies to PLK4 (1:200 dilution) and actin (Sigma, A2066; 1:10,000 dilution).

**Zebrafish studies.** Adult male and female zebrafish (<18 months old) from wild-type (AB Tübingen) and transgenic strains were maintained under standard laboratory conditions on a 14-h light/10-h dark cycle at 28 °C. Experiments were performed in compliance with ethical regulations under UK Home Office license PL 60/4418. Adult pairs were used to generate embryos at 0–5 d.p.f., and the signal over all the sections was summed. The intensity values for a cytoplasmic background of matched volume were obtained and subtracted from the centrosome signal.

**FACS analysis.** For FACS analysis, embryos were dechorionized with pronase (2 mg/ml), dissociated into single-cell suspension (as described for cell counting) and fixed in 4% paraformaldehyde. Mitotic cells were then labeled with antibody to phosphorylated histone H3 (Millipore, 06-570; 1:500 dilution), and DNA content was assessed by staining with propidium iodide (50 μg/ml). Apoptotic cells were labeled live with Annexin V (Life Technologies, A13201; 1:20 dilution), with propidium iodide (3.5 μg/ml) used as a counterstain to identify viable cells. Cells were sorted on a BD Biosciences FACSaria II, and data were analyzed using FlowJo software (v7.6.1, Tree Star).

**Statistical analysis.** All data are shown as averages, with variance either as s.e.m. or s.d. Statistical analysis was performed using Prism (GraphPad Software). For all quantitative measurements, a normal distribution was assumed, with t tests performed, unpaired and two-sided unless otherwise stated. For categorical data, a χ² test was used. Data collection and analysis were not performed with blinding, as the different conditions were clearly recognizable to the experimenter, aside from zebrafish surface area determination. No statistical methods were used to predetermine sample sizes, which were determined empirically from previous experimental experience with similar assays and/or from sizes generally employed in the field.