The Zebrafish *curly fry* Is Required for Proper Centrosome and Mitotic Spindle Assembly

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Abstract

The zebrafish *curly fry* (*cfy*) mutation leads to a dramatic increase in mitotic index and cell death starting during neural tube formation. The mutant phenotype is cell autonomous and does not result from defects in apical/basal polarity within the neuroepithelium. The increase in mitotic index could be due to increased proliferation or cell cycle arrest in mitosis. *cfy* embryos were analyzed to examine these two possibilities. By labeling embryos with a pulse of BrdU and anti-phospho-histone 3 and examining the DNA content by fluorescence activated cell sorting, we show that *cfy* mutants exhibit no increase in proliferation, but a significant increase in the number of cells arrested in mitosis. Furthermore, time-lapse microscopy *in vivo* confirmed that a great majority of dividing cells arrest during mitosis and that these mitotically arrested cells die in *cfy* embryos. Finally, immunostaining and confocal microscopy in *cfy* mutant embryos revealed that mitotic cells in mutants contain aberrant centrosomes and often exhibit monopolar spindles, thereby leading to mitotic cell cycle arrest. Our results suggest that the *cfy* gene is required for proper centrosome assembly and mitotic spindle formation, therefore critical for normal cell division.

Keywords: mitosis, cell cycle, proliferation, bipolar spindle, centrosome, *Danio rerio*

Introduction

CENTROSOMES WERE IDENTIFIED in the late 19th century as organelles pivotal for mitosis.¹ Centrosomes consist of a pair of centrioles surrounded by a matrix of pericentriolar material (PCM) that is responsible for nucleation of microtubules during cell division in metazoans.² The centrosome is duplicated once per cell cycle, starting at G1/S-phase. As the cell cycle progresses to mitosis, duplicated centrosomes increase in size by recruiting PCM through a process called centrosome maturation. To sustain the fidelity of cell division, the centrosome must duplicate only once per cell cycle and only one daughter per mother. Errors in this process result in too few or too many centrosomes, causing chromosome mis-segregation and aneuploidy. It has been shown that dysregulation of centrosome assembly associates with human diseases, including cancer, ciliopathy, and microcephaly.^{3–7}

Molecular and genetic studies in diverse systems have uncovered molecular mechanisms of centrosome assembly through the identification of molecular players required for normal centrosome duplication and maturation during the cell cycle.² Centrosome duplication is triggered by the action

of cell cycle regulators such as cdk2/cyclinE⁸ and also requires ϵ -tubulin⁹ in *Xenopus* egg extracts, centrin-2 in mammalian cells,¹⁰ and the kinase ZYG-1 and coiled-coil proteins SAS-4, SAS-5, SAS-6, and SPD-2 in *Caenorhabditis elegans*.^{11–18} The regulatory mechanisms of centrosome assembly appear to be highly conserved among vertebrates, flies, and nematodes.² For example, Plk4/ZYG-1,^{19,20} CPAP/SAS-4,^{21,22} STIL/SAS-5,²³ HsSAS-6/SAS-6,¹⁵ and Cep192/SPD-2^{24–26} are shown to be required for proper centrosome assembly in a conserved manner among the phyla. Furthermore, maturation of centrosomes involve additional players, including the action of SPD-5,²⁷ Cep192/SPD-2,^{13,18,24,25} Aurora A kinase,^{28–30} polo kinases,^{31–33} and γ -tubulin.³⁴ However, some factors such as Cep152 and centrin-2 are found to be required for centrosome assembly in vertebrates,^{10,25,35} but are absent in the *C. elegans* genome.

Despite current advances in the regulation of centrosome assembly, our understanding of the molecular underpinnings of centrosome duplication and maturation in vertebrates remains incomplete. In this regard, further investigation of centrosome regulation in vertebrates would be useful. Our previous work reported a dramatic increase in the number of

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mitotic cells and cell death in *curly fry* (*cfy*) embryos starting at the neurulation stage, as well as reduced neural progenitor pool.³⁶ At later stages, fewer neurons and oligodendrocytes were observed in the mutant central nervous system (CNS), suggesting that mitotic cells might die. We also showed that deregulation of cell division was not due to defective polarity of the neuroepithelium and that abnormal cell division arose cell autonomously. In this study, we analyzed proliferation and cell cycle of the zebrafish *curly fry* (*cfy*) mutant embryos. We found in mutants that proliferation was unchanged, but cells arrested in mitosis. Mitotically arrested cells exhibited monopolar spindles with abnormal centrosomes, suggesting that the *cfy* gene product is required for normal centrosome function.

Materials and Methods

Embryos

The homozygous *cfy* mutation arose spontaneously within one breeding colony of zebrafish obtained from the Nüsslein-Volhard laboratory in Tübingen, Germany. Embryos were obtained from natural spawnings of adult fish kept at 28.5°C on a 14-h light/10-h dark cycle and were staged according to Kimmel *et al.*³⁷ Embryos older than 24 hours postfertilization (hpf) were raised in 0.003% 1-phenyl-2-thiourea (Sigma) in egg water to inhibit the production of pigment. For time-lapse movies, embryos were manually dechlorinated and kept in 2% low melting agarose gel (in 1×Evans) supported by a coverslip.³⁸ For necessary stages, embryos were immobilized with tricaine³⁹ (Sigma). For time-lapse movie, *H2A.F/Z* histone GFP transgenic fish⁴⁰ were crossed with *cfy* heterozygotes and their F1 progeny were identified for *cfy*/+; *H2A.F/Z*. Embryos from a cross between a pair of *cfy*/+; *H2A.F/Z* were scanned under confocal microscopy (LSM510; Zeiss) using the protocols for making time-lapse videos of dividing cells.^{38,40,41}

Fluorescence activated cell sorting analysis

Thirty embryos were collected for wild-type and *cfy* mutants at 24 hpf. Embryos were genotyped by their brain ventricle and body curvature phenotype. Embryos were dechlorinated by pronase (2 mg/mL in embryo medium), immersed in calcium-free Ringer's solution³⁹ for 30 min, and dissociated into single cells using trypsin (ICN Biochemicals, Inc.) and pipetting embryos several times. Dissociated cells were resuspended in a cell culture medium Dulbecco's modified Eagle's medium (DMEM) +20% fetal bovine serum, passed through meshes 105 then 40 (μmesh; Small Parts, Inc.), and spun down at 1200 rpm for 10 min to isolate single cells. To get rid of cell debris, cell pellets were resuspended with 0.01% tween-20 in phosphate-buffered saline and spun down (repeated twice). Collected cells were resuspended in propidium iodide (0.05 mg/mL propidium iodide in a solution of 0.1% sodium citrate and 0.01% Tween-20) to label nucleic acid and treated with RNase A to destroy RNA. Cells with their DNA labeled with propidium iodide were sorted in a flow cytometer (FACSVantage SE Sorter), and the propidium iodide fluorescence per cell was measured. DNA contents and cell cycle were analyzed using the software MultiCycle (Phoenix Flow Systems).

Immunostaining

For antitubulin labeling, embryos were fixed in 37% FPA:n-heptane (1:1) for 1 h by gentle shaking at room temperature (RT) to stabilize cytoskeletal structures. For other antibody staining, embryos were processed as described previously.³⁶ We used the following antibodies: polyclonal rabbit anti-phospho histone H3 (anti-PH3; Upstate Biotechnology, 1:1000), monoclonal anti-γ-tubulin (Sigma; 1:500), and monoclonal anti-α-tubulin (Sigma; 1:500). We used secondary antibodies conjugated to Alexa Fluor 488 or -568 (Invitrogen; 1:2500). Embryos were sectioned by 10 μm thickness using a Leica VT1000S vibratome. Incubations and washes for antibody stainings were performed on floating sections in blocking solution. Stained sections were coverslipped and analyzed on a Zeiss Axiophot DIC microscope. Fluorescence images were captured using LSM 510 confocal microscope and processed with Photoshop software. To measure the width of centrosome, confocal images of γ-tubulin labeling were exported as TIFF files and analyzed using NIH-image software.

BrdU-incorporation and labeling

Embryos were incubated for 2 h in BrdU (10 mM/4% dimethyl sulfoxide in embryo medium) starting at 22 hpf.⁴² After incorporation, embryos were rinsed for 10 min in embryo medium and fixed. Embryos were then cryosectioned. DNAs were denatured with 0.2 N HCl for 2 h at RT and neutralized with 0.1 M sodium borate (pH 8.5) for 30 min. Treated sections were immunostained with anti-BrdU (Serotec, Inc.; 1:500) and anti-PH3 and were visualized with secondary antibodies (Molecular Probes; Alexa Fluor 488 or -568, 1:2500). Confocal images were taken for each section, and cells labeled with anti-BrdU and/or anti-PH3 were counted for cell cycle analysis. To calculate the percentage of the total number of cells that were labeled by BrdU in the sections, all nuclei in serial sections of comparable areas of wild-type and *cfy* embryos were stained with DAPI. For each data set, the mean ± standard deviation was calculated.

Tau:DsRed construct and DNA injections

For the tau:DsRed construct, a fragment encoding bovine tau was amplified by PCR from the tau:EGFP construct³⁸ using the primer pair, 5'*Hind*III-tau (5'-TGAACCAGGATG GCTGAGCCCCGCCAGGAGTTC-3') and 3'*Sal*III-tau (5'-TGGTCGACCCGGACACGATCTCCGCCCCGTG-3'). The amplified fragments were digested with *Hind*III and *Sal*I and cloned into the pXEX-DsRed construct.⁴³ Reconstructed tau:DsRed plasmid was sequenced to confirm that these fusion proteins are in-frame. For time-lapse movie, 10 ng/μL DNA was injected into 1–2 cell stage embryos from either *cfy* heterozygote or *cfy*/+; *H2A.F/Z* crosses.

BODIPY-ceramide labeling

BODIPY-FL-C5-Ceramide (D-3521; Invitrogen) stock solution was made at 500 μM. 20 μM final concentration was applied to 9 hpf embryos in darkness at 28.5°C overnight. The embryos were rinsed thoroughly in embryo medium and dechlorinated before mounting for time-lapse microscopy.⁴⁴

Results

The cfy embryo exhibits cell cycle arrest in mitosis

We have previously observed a significantly increased mitotic index in *cfy* mutants.³⁶ The increased mitotic figures in *cfy* mutant embryos might result from increased cell proliferation. To examine cell proliferation, we quantified the number of cells that incorporate BrdU in *cfy* mutants. Embryos at 22 hpf were exposed to 10 mM BrdU for 2 h, which is likely less than the duration of one cell cycle during these developmental stages,^{45–47} and fixed right after the pulse of BrdU (see Materials and Methods section). Cells that entered S-phase at least once during the BrdU pulse should be BrdU positive. BrdU embryos were also labeled with anti-PH3 to mark cells in mitosis. In our prior study, we observed a significantly increased mitotic index in the neural tube of *cfy* embryos compared with wild-type sibling embryos at this stage.³⁶ If an increase in proliferation accounted for an increase in mitotic index, then the proportion of BrdU-positive cells should have increased in *cfy* embryos compared to wild-type sibling embryos. If cells were arrested in mitosis, the number of BrdU-positive cells should be unaffected, or possibly decreased in the mutants.

Sections through the neural tube were examined for both anti-BrdU-positive and anti-PH3-positive cells both in wild-type sibling and *cfy* embryos at 24 hpf (Fig. 1). In *cfy* embryos, there was a dramatic increase in the number of cells in mitosis, as previously observed.³⁶ Quantification of mitotic figures revealed that *cfy* embryos exhibited a 10.1-fold increase in the number of mitotic cells in the brain and 3.8-fold

TABLE 1. QUANTIFICATION OF BRdU-LABELED CELLS IN THE CENTRAL NERVOUS SYSTEM

	Brain	Spinal cord
wt	93.9 ± 31.3 (n = 29)	24.7 ± 15.8 (n = 14)
<i>cfy</i>	61.8 ± 24.7 (n = 21)	6.5 ± 4.1 (n = 11)
ratio (<i>cfy</i> /wt)	0.66	0.26
p-Value (wt vs. <i>cfy</i>)	<0.001	<0.001

n = number of sections counted for five embryos (average ± SD). SD, standard deviation; wt, wild type.

increase in the spinal cord, whereas there was a significant decrease in the number of BrdU-labeled cells in sections of both the brain and spinal cord (Table 1). The ratio of BrdU-labeled cells between *cfy* and wild-type embryos was 0.66 in the brain and 0.26 in the spinal cord of *cfy* embryos compared with those of wild-type siblings. The different effect on the ratio of BrdU-labeled cells between the brain and spinal cord may suggest the tissue specific regulation of cell proliferation. In support of this tissue specificity, we have shown tissue dependent differences in the ratio (*cfy*/wt) of the mitotic index in our prior study.³⁶ Furthermore, in comparable sections of the CNS, there was no significant difference in the total number of cells labeled with DAPI between *cfy* (194.3 ± 13.7, n = 5) and wild-type sibling embryos (195.3 ± 12.7, n = 5). Thus, compared to wild-type siblings, *cfy* embryos exhibited a reduced number of BrdU-positive cells but no change in the total number of cells, suggesting that cell

FIG. 1. In *cfy* embryos, many mitotic cells are BrdU negative, whereas all the mitotic cells in wild-type embryos are BrdU positive. Horizontal sections through the hindbrain of wild-type sibling (A) and *cfy* (B) embryos at 24 hpf. Embryos were labeled with anti-BrdU (red) and anti-PH3 (green). In wild-type, all the anti-PH3 labeled cells were also anti-BrdU labeled. In contrast, only a fraction of anti-PH3 labeled *cfy* cells were also anti-BrdU labeled. Note that the *cfy* embryo exhibits an increased mitotic index marked by anti-PH3, but fewer cells were labeled with anti-BrdU. Arrows indicate cells colabeled with anti-PH3 and anti-BrdU. (A, B) Insets for box a and b are magnified 4× and 1.5× folds, respectively. Dorsal is up. Scale bar, 20 μm. hpf, hours postfertilization.

TABLE 2. COMPARISONS OF MITOTICALLY ARRESTED CELLS VERSUS PROLIFERATING CELLS

	Brain			Spinal cord		
	Total number of PH3 labeled	Both PH3 and BrdU	Only PH3 labeled	Total number of PH3 labeled	Both PH3 and BrdU	Only PH3 labeled
wt	6.7 ± 3.8 (n=29)	6.7 ± 3.8 (n=29)	0 (n=29)	2.8 ± 1.8 (n=14)	2.8 ± 1.8 (n=14)	0
cfy	67.5 ± 24.9 (n=21)	15.2 ± 7.8 (n=21)	52.3 ± 20.9 (n=21)	10.6 ± 2.5 (n=11)	1.3 ± 0.9 (n=11)	9.4 ± 2.6 (n=11)

n = number of sections counted for five embryos both in wild-type and *cfy* embryos (average ± SD).

proliferation is not increased in *cfy* mutants. Therefore, increased proliferation is unlikely to be responsible for the increase in mitotic index observed in *cfy* embryos.

Next, we examined if mitotic cells arrest in *cfy* embryos, by quantifying the number of anti-PH3-positive cells in comparison with anti-BrdU-positive cells at 24 hpf (Table 2). In wild-type siblings, all the anti-BrdU-positive cells were also anti-PH3-positive (Fig. 1A), indicating that all the mitotic cells at the time of fixation entered S-phase of the cell cycle during the 2 h period of BrdU-exposure. In contrast, there was a large number of anti-PH3-positive cells that were anti-BrdU-negative in *cfy* embryos (Table 2). Cells that were anti-PH3-positive but anti-BrdU-negative should represent cells that were in mitosis but had replicated their DNA before BrdU-exposure, consistent with mitotic arrest. In the brain of *cfy* embryos, only 22.6% (15.2/67.5) of anti-PH3-positive cells were also anti-BrdU-positive, while 77.4% (52.3/67.5) of anti-PH3-positive cells were anti-BrdU-negative (Table 2). Thus, the majority of anti-PH3-positive cells in *cfy* embryos may have stalled in mitosis. Taken together, our data suggest that mitotic cell cycle arrests likely account for the increased mitotic index observed in *cfy* mutants.

Fluorescence activated cell sorting analysis confirms no increase in cell proliferation, but cell cycle arrest in cfy embryos

Increased frequency of cell division or cell cycle arrest should affect the proportion of cells in G1, S, and G2/M phases during cell cycle progression. To determine the proportion of cells in G1 (2 N DNA), G2/M (4 N DNA), and S (between 2 N and 4 N DNA) phases, we measured the amount of DNA in cells from *cfy* and wild-type sibling embryos (24 hpf) by fluorescence activated cell sorting (FACS) analysis. If the frequency of cell division increased in *cfy* embryos, there should be an increase in the S-phase population compared to wild-type embryos. If cells arrested in mitosis, the proportion of cells in G2/M-phase should be higher in mutant embryos compared to wild-type embryos. Our FACS analysis revealed that there was no difference in the proportion of cells in S-phase (~30%) in both *cfy* and wild-type

siblings (Table 3). However, we observed a 1.7-fold increase in the proportion of cells in G2/M-phase in *cfy* mutants compared to wild-type siblings and a decrease in the proportion of cells in G1-phase. Therefore, our FACS analysis suggests that cell proliferation does not increase in *cfy* embryos, but that cells appear to accumulate in G2/M, corroborating the conclusions from quantification of anti-BrdU and anti-PH3 labeling.

Time-lapse microscopy suggests that cells in cfy embryos likely arrest in mitosis

Time-lapse video microscopy of dividing cells allows one to determine whether there is an increase in the number of dividing cells or cell arrest. For these experiments, we used a transgenic line of zebrafish expressing a fusion of a histone protein variant, H2A.F/Z, to green fluorescent protein (H2A.F/Z:GFP).⁴⁰ In these transgenic embryos the fusion proteins are localized to the nuclei, facilitating observation of the dynamics of dividing cells in living embryos (Fig. 2).^{38,40,41} To follow cell cycle progression in *cfy* embryos, a line of zebrafish that is transgenic for H2A.F/Z:GFP and heterozygous for *cfy* was generated by raising progeny from crosses between the homozygous (H2A.F/Z:GFP) transgenics and *cfy* heterozygotes. Sexually mature H2A.F/Z:GFP expressing *cfy* carriers were identified by its ventricle phenotype and expression of H2A.F/Z:GFP. Embryos from a cross between identified transgenic *cfy* carriers were used for time-lapse analysis.

To examine cell cycle progression in a living embryo, we observed morphological changes on H2A.F/Z:GFP fusion protein expression at a single cell level. Dividing cells in each embryo were examined blindly at earlier stages, and the genotype of each embryo was identified at 24 hpf. As reported previously,³⁶ our time-lapse microscopy of living *cfy* embryos confirmed the onset of cell division phenotype starting at 12–14 hpf, as well as the morphological phenotypes at 24 hpf. At these stages, *cfy* mutant embryos exhibited an increased mitotic index and cell deaths.³⁶ In *cfy* embryos, an increased number of mitotic cells were evident in time-lapse movies that were visualized by H2A.F/Z:GFP fusion protein expression (Fig. 2B), whereas wild-type transgenic embryos exhibited normal cell cycle progression (Fig. 2A). In wild-type embryos, a cell entering mitosis, which was identified by chromosome condensation, underwent progressive morphological changes of mitotic phases and completed cell division to produce two daughter cells within 20 min (Supplementary Movie S1A; Supplementary Data are available online at www.liebertpub.com/zeb). However, in *cfy* transgenic embryos (Fig. 2B), a cell entering mitosis failed to undergo morphological changes of successive mitotic phases, but

TABLE 3. QUANTIFICATION OF CELL CYCLES

	G1 (%)	S (%)	G2/M (%)
wt (n=4)	58.8 ± 5.9	30.0 ± 6.7	11.3 ± 1.1
cfy (n=4)	51.2 ± 4.1	30.4 ± 3.4	18.8 ± 0.6
ratio (cfy/wt)	0.87	1.01	1.7

n = number of independent experiments (average ± SD).

FIG. 2. Time-lapse microscopy directly demonstrates mitotic arrest in *cfy* embryo. Dorsal views of the hindbrain of live wild-type sibling (A) and *cfy* (B) embryos expressing H2A:GFP (see Supplementary Movie S1A, B). Numbers in the lower right-hand are elapsed time (in seconds) of selected time points from the time-lapse movies starting from the first sign of chromosome condensation in cells tracked (arrowhead in A, and arrow in B). In the wild-type embryo (A), a cell (arrowhead, $t=0$ s) that entered mitosis completed a cell division and produced two daughter cells (arrowheads, $t=1192$ s) within 20 min. In the *cfy* embryo (B), a cell that entered mitosis (arrow, $t=0$ s) was stalled in mitosis throughout the time-lapse movie (arrow, $t=3728$ s). Note that the number of cells with condensed chromosomes increases in the midline of the neural tube during the time-lapse movie. Arrowhead and arrow in the panel (B) denote two cells tracked in this movie. Anterior is left. Scale bar, $50\ \mu\text{m}$.

remained as circular morphologies of condensed chromosome over several hours (Supplementary Movie S1B). Once cells entered mitosis, they were stalled in mitosis and these mitotic cells accumulated at the ventricular zone in *cfy* embryos as development proceeded. This observation could explain that mitotic index increases at 24 hpf compared to earlier stages starting from 12 to 14 hpf in *cfy* embryos as shown in the prior study.³⁶

Time-lapse movies were recorded within a region of $184.3 \times 184.3\ \mu\text{m}$ in the dorsal hindbrain of embryos using confocal microscopy. Wild-type embryo was recorded for time-lapse movie for 157 min in 42 s intervals. There were 75 mitotic cells observed during the time-lapse movie and all the mitotic cells exhibited normal progress of mitotic cell cycles. Using these time-lapse movies, we measured the duration of cell cycle from prophase to telophase ($n=30$ mitotic cells), which was observed to be 737.8 ± 87.3 s. We also recorded live *cfy* embryos for 170 min in 42 s intervals. During this time, 101 mitotic cells were identified but only 7 mitotic cells completed cell divisions and produced two daughter cells, suggesting that in *cfy* embryos, more than 90% of cells entering mitosis arrested. The duration of cell cycle from prophase to telophase was also measured for these seven mitotic cells (756 ± 59.3 s) and found to be comparable to that of wild-type cells. However, the rest of the mitotic cells never completed cell division over the period of 170 min of time-lapse movies. Once chromosomes were condensed, they resided in a circular morphology throughout the period of time-lapse imaging. Most of the cells in mitosis actively moved around

and kept changing their focal planes during the recording, suggesting that these cells might be still alive. Some of mitotic cells that stayed on the same focal plane clearly demonstrated a significantly increased duration in mitosis. The longest period observed was 117.6 min in mitosis with no apparent progression in the mitotic phase. Thus, live imaging directly revealed that cells in *cfy* embryos arrested in mitosis.

Time-lapse microscopy reveals that mitotically arrested cells undergo cell death

Previous study showed that *cfy* embryos exhibit a dramatically increased mitotic index, as well as an increased cell death measured by anti-cleaved caspase 3.³⁶ Using time-lapse microscopy, we further investigated if mitotically arrested cells led to cell death. We injected embryos with a DNA construct expressing the microtubule binding protein, Tau, fused with DsRed so that mosaically labeled cells could be tracked throughout time-lapse imaging. To identify individual cells, we also labeled the cell plasma membrane with BODIPY dye⁴⁸ (Fig. 3). Wild-type cells that expressed Tau:DsRed displayed bipolar spindles and completed normal cell divisions (Fig. 3A). In contrast, *cfy* cells that expressed Tau:DsRed exhibited monopolar spindles throughout the time-lapse movie. Furthermore the mutant cells exhibited membrane blebs and disintegrated (Fig. 3B), which is consistent with cell death of the mitotically arrested cells. Previous reports indicate that cell rounding and blebbing are characteristic of the final stage of apoptosis.^{49,50} Thus,

FIG. 3. Time-lapse microscopy suggests that mitotically arrested cells die in *cfy* embryos. Dorsal views of the hindbrain of wild-type sibling (A) and *cfy* (B) embryos expressing the microtubule binding protein, Tau:DsRed (red) in a mosaic manner. In both embryos, cell membranes were labeled with BODIPY dye (green), and the wild-type embryo (A) expresses H2A:GFP (green). Numbers in the lower right-hand are elapsed time of selected time points from time-lapse movie. In the wild-type embryo (A), a cell (arrow, $t=0$ s) that entered mitosis divided into two daughter cells (two arrows, $t=720$ s). Note mitotic cells (arrow and arrowhead) in the wild-type embryo (A) exhibited bipolar spindles visualized by Tau:DsRed (red). In the *cfy* embryo (B), a cell (arrow, $t=0$ s) halted in mitosis and exhibited monopolar spindles that were visualized by Tau:DsRed (red). Cells in mitosis are marked by round-shaped cell membranes. This mitotically arrested cell (arrow) became fused with a neighboring cell (arrowhead). Note a lot of blebs of cell membranes, suggestive of cell deaths. Anterior is left. Scale bar, 50 μ m.

time-lapse microscopy suggests that the increase in cell death observed in mutants³⁶ may be due to mitotic arrest of dividing cells.

cfy mutant embryos exhibit circular mitotic figures

The quantification of BrdU- and PH3-labeled cells, FACS analysis, and time-lapse movies suggested that the vast majority of cells in *cfy* embryos arrest in mitosis. We thus asked what might cause this mitotic cell cycle arrest in *cfy* mutant embryos. At the onset of mitosis, the nuclear envelope breaks down, the nuclear lamina depolymerizes, and interphase chromatin condenses to chromosomes. Concomitantly, cytoplasmic microtubules are reorganized into bipolar mitotic spindles, a highly dynamic structure required for the accurate segregation of sister chromatids.⁵¹ To examine mitotic cells, embryos were first stained with anti-PH3 for a mitosis marker and anti- α -tubulin to label mitotic spindles (Fig. 4). Non-arrested and actively dividing cells should be found randomly in all phases of the mitotic cell cycle. In contrast, arrested cells may accumulate at a particular phase of the mitotic cell cycle. As expected in wild-type siblings (Fig. 4B), mitotic figures were found at different stages of mitosis (56.7% in prophase, 22.7% in metaphase, and 20.6% in ana/telophase of 97 mitotic figures in 10 embryos). However, the great ma-

jority of mitotic figures in *cfy* embryos were circular and hypercondensed with the absence of the conventional anaphase and telophase figures (Fig. 4D). Mitotic spindles in *cfy* embryos were nucleated by an apparent single pole with the microtubules arrayed radially in the center and condensed chromosomes at the periphery. Thus, abnormal spindles with circular mitotic figures in mutant cells are likely the reason for cell cycle arrest in mitosis.

Mitotic cells in cfy embryos exhibit monopolar spindles with aberrant centrosomes

We observed that the *cfy* embryo exhibited monopolar spindles in mitotic cells (Fig. 4D), in contrast to wild-type mitotic cells that showed bipolar mitotic spindles (Fig. 4B). These observations provide a possible mechanism by which many cells in *cfy* embryos arrest in mitosis. Monopolar spindles may result from a defect in centrosome duplication, maturation, or separation. To examine centrosome assembly, we immunostained embryos with anti- γ -tubulin to label centrosomes to determine the distribution of γ -tubulin foci in mitotic cells that were colabeled with anti-PH3 (Fig. 5). In all wild-type mitotic cells ($n=39$, Fig. 5A), two distinct foci of γ -tubulin were separated by more than 5 μ m and localized on either side of the chromosomes. Remarkably, the great

FIG. 4. Mitotic cells in *cfy* embryos display monopolar spindles. Cross-sections of wild-type (A, B) and *cfy* (C, D) brains were labeled with the mitotic marker, anti-PH3 (green), and anti- α -tubulin (red) for microtubules. (A) Magnified (4 $\times$) view of microtubules of a wild-type mitotic cell in brain (B) illustrates bipolar spindles (arrowheads) at anaphase. In contrast, (C) magnified (4 $\times$) view of microtubules of a mitotic cell in *cfy* brain (D) exhibits monopolar spindles (arrows). Scale bar, 10 μ m.

majority of mitotic cells (84% = 56/67) in *cfy* embryos exhibited a single γ -tubulin focus (Fig. 5B, C), suggesting that many cells in *cfy* mutant embryos failed to properly duplicate centrosomes. While a small portion (16%) of mitotic cells in *cfy* embryos exhibited two γ -tubulin foci (Fig. 5D, E), these two foci were separate by less than 1 μ m and both were found at the center of the cell. This observation suggested that centrosome separation might be impaired in *cfy* embryos as well. In addition, in cases of cells with two foci in mutant

embryos we sometimes observed that one centrosome was bigger than the other (11/67), which might be indicative of abnormal centrosome maturation. This is similar to the one normal and one smaller centrosome due to impaired centriole structure exhibited by mitotic cells in *C. elegans* with a partial depletion of SAS-4, SAS-5, SAS-6, and ZYG-1, which are components of centriole duplication.^{12,14,15}

To get a better understanding of the centrosome defects seen in *cfy* cells with a single γ -tubulin focus, we measured

FIG. 5. *cfy* embryos exhibit aberrant centrosomes. (A–E) Cells are colabeled with anti-PH3 (green) for mitotic cells and with anti- γ -tubulin (red) for centrosomes. Insets are 4 $\times$ magnified views of centrosomes. In wild-type (A), duplicated centrosomes separate to opposite sides of the cell. In the majority of *cfy* embryos (84%, $n = 67$ centrosomes), only a single focus of γ -tubulin labeling was apparent (B, C). A small portion of mitotic cells in *cfy* embryos (16%, $n = 67$ centrosomes) had two foci (arrows) of γ -tubulin, often with one bigger and another smaller centrosome (D), or two smaller centrosomes (E). These asymmetric centrosomes failed to separate properly and were located at the center of condensed chromosomes (D, E). Scale bar, 10 μ m. (F) Histograms showing the width (diameter, μ m) of γ -tubulin foci in wild-type sibling ($n = 78$ centrosomes) and *cfy* ($n = 67$ centrosomes) embryos. The width of wild-type centrosomes ranges from 0.6 to 1 μ m, however, *cfy* centrosomes exhibit a wider range with the widths from 0.1 to 1.769 μ m.

the size of the single centrosome (Fig. 5F). If centrosomes duplicated normally but failed to separate, then the single focus should have a diameter larger compared with a separated centrosome in wild-type cells.^{30,52} If centrosomes fail to duplicate, the diameter of centrosomes should be the same as that of a separated centrosome in wild-type cell. Interestingly, we found that the diameter of each centrosome in *cfy* mitotic cells ranged broadly from 0.1 to 1.769 μm , with 50% 0.6–1 μm in diameter, 14% <0.6 μm in diameter, and 36% > 1 μm in diameter (Fig. 5F). In contrast, the diameter of wild-type mitotic cells ranged more tightly with 91% from 0.6 to 1 μm in diameter, 3% <0.6 μm in diameter, and 6% > 1 μm in diameter (Fig. 5F). Using 0.6 to 1 μm as the diameter of a single separated centrosome in a wild-type cell, it appears that at least 50% of the mitotic cells in *cfy* embryos failed to duplicate their centrosome. Furthermore, the fact that 14% of *cfy* centrosomes were <0.6 μm in diameter compared to 3% of wild-type centrosomes may indicate a defect in centrosome maturation and that 36% of *cfy* centrosomes were >1 μm in diameter compared with 6% of wild-type centrosomes may indicate impaired centrosome separation. Thus, it is possible that defects in centrosome duplication, maturation, and separation may all contribute to the aberrant centrosome assembly in *cfy* mutants.

The cfy gene is not knsl1, aurora B, nor the gene for STIL/SIL

Large-scale insertional mutagenesis screening identified several known cell cycle regulators in zebrafish.⁵³ Based on our phenotypic analyses on centrosome defects and monopolar mitotic spindles, we examined whether defects in some genes known to be important for centrosome assembly might be causal for the *cfy* mutation. One gene examined was zebrafish *kns11* that encodes the kinesin-related motor protein Eg5, which is known to function in centrosome separation and bipolar spindle assembly in frog^{54,55} and human cell lines.⁵² We performed a complementation test and found that the *cfy* and the *kns11* (hi 486) complemented each other, which rules out the possibility that the *cfy* could be a zebrafish *eg5* homolog. We also tested the zebrafish mutant of an *aurora B*-like kinase homolog (hi 1046), finding that hi 1046 complemented with the *cfy* to exclude a mutation in the *aurora B*-like kinase as causal for the *cfy*. In zebrafish, the phenotype of loss-of-function mutations in the centriole component STIL/SIL also resembles that of *cfy* mutants.^{36,56} However, STIL/SIL maps to LG 22, whereas our current mapping data place the *cfy* to LG 17 (data not shown), suggesting that the *cfy* mutation affects a different gene. Thus the *cfy* gene is not *kns11*, *aurora B*, nor the gene for STIL/SIL.

Discussion

The cfy mutation leads to aberrant centrosome assembly and monopolar spindles, leading to mitotic cell cycle arrest

Two mechanisms could account for the increased mitotic index in *cfy* mutant cells. There could be an increase of proliferation in mutant embryos, or dividing cells might arrest during mitosis. Our analyses in *cfy* embryos suggest that

the increase in mitotic index results from cell cycle arrest, not from increased cell proliferation. First, the proportion of anti-PH3-positive cells was significantly increased in *cfy* embryos; however, the majority of anti-PH3-positive cells were BrdU negative. Second, *cfy* mutants did not exhibit an increased number of BrdU-positive cells. Third, FACS analysis in *cfy* mutants revealed an increase in the proportion of cells that are in G2/M-phase but not in S-phase, compared to wild-type siblings. Finally, time-lapse analysis directly demonstrated that the great majority of dividing cells in mutants arrested as they entered mitosis, while all cells divided normally in wild-type siblings.

Our cytological analysis revealed that the majority of mitotic cells labeled with anti-PH3 have circular mitotic figures in *cfy* mutant embryos, while mitotic cells in wild-type embryos display mitotic figures in all phases. In fact, mitotic cells in *cfy* embryos exhibited monopolar spindles with the microtubules arrayed radially in the center and condensed chromosomes at the periphery (84%). A small portion (16%) of *cfy* mitotic cells displayed two distinct γ -tubulin foci. These two foci were highly asymmetric and irregular in size and resided within a single centrosome, likely leading to a single functional microtubule-organizing center (MTOC). In wild-type cells, however, two distinct centrosomes organized bipolar mitotic spindles. Thus the morphological analysis of centrosomes in *cfy* embryos suggests that the *cfy* gene plays a critical role in centrosome assembly and mitotic spindle formation, thus forming proper cell division.

Defective centrosome assembly may lead to abnormal CNS development in cfy mutants

Abnormal centrosome assembly is known to be associated with human diseases, including cancer, ciliopathy, and microcephaly.^{3–7} Centrosome amplification often leads to multipolar mitotic spindles, genomic instability, and cell death. While supernumerary centrosomes often cluster to form bipolar spindles, the centrosome clustering process is error prone, often resulting in chromosome missegregation and aneuploidy.⁵⁷ Furthermore, loss-of-function mutations in centrosome factors are linked to human primary microcephaly. Primary microcephaly results from a reduction in the total number of neural progenitor cells leading to a reduced brain size.^{7,58} Deregulated centrosome function might disrupt the balance of symmetric and asymmetric cell divisions in the neural progenitor cells, which could affect the pool of neural progenitor cells.^{7,59} Another possibility is that disrupted cell division could lead to increased apoptosis, decreasing the total population of neural progenitor cells. The latter is supported by studies in zebrafish, where mutations in the centrosome factor *cassiopeia*/STIL,⁵⁶ knockdown of the Plk4 kinase,²⁰ or the centrosome component NEDD1⁶⁰ result in a smaller brain size, likely due to increased apoptosis in the developing brain. Consistent with these findings, our previous work found that the *cfy* mutation results in increased apoptosis, a reduced number of neural progenitor cells, and smaller brain size in zebrafish embryos.³⁶ In this study, we show that the *cfy* mutation results in aberrant centrosome assembly and mitotic arrest, which likely lead to cell death and a size reduction of the developing brain. Thus, the *cfy* mutant may be a useful tool to study a human disorder, primary microcephaly, that is closely linked to centrosome regulation.

Molecular identity of the *cfy* gene

Although the molecular identity of *cfy* is unknown, it may be useful to speculate on what sort of gene *cfy* might be. One key feature of the *cfy* mutation is that the mitotic phenotype is cell autonomous, and normal *cfy* function is required in dividing cells.³⁶ Two proteins known to be required for normal mitosis are the BimC kinesin, Eg5,⁶¹ and the protein kinase, Aurora B.⁶² Both are associated with centrosomes and *eg5* loss-of-function results in monopolar spindles due to failure of duplicated centrosomes to separate properly in a variety of organisms.^{52,54,63} However, *cfy* is neither *eg5* nor *aurora B* since carriers for zebrafish insertional mutations for these genes⁵³ do complement *cfy* carriers.

The *cfy* gene could encode an important component of centrioles. In zebrafish, loss-of-function mutations in the centriole component STIL/SIL result in the *cassiopeia* phenotype⁵⁶ that resembles phenotypes observed in *cfy* mutants. Both *cfy* and *cassiopeia* mutants have an increased mitotic index, ventral curvature, and decreased number of neural progenitor cells.^{36,56} However, STIL/SIL does not map to our current mapping position, suggesting that the *cfy* mutation affects a different gene, likely with a similar biological role as STIL/SIL in centriole structure and duplication.

Other candidates for the *cfy* gene include Plk4 that is required for centrosome duplication in vertebrates.^{19,20} In humans, loss-of-function mutations in Plk4 are also reported to result in primary microcephaly, reminiscent of the reduced neural progenitor pool observed in *cfy* and *cassiopeia* mutant embryos.^{20,36,56} The *cfy* protein could be another serine-threonine kinase associated with centrosomes, Plk1, which is important for centrosome maturation in human cells.⁶⁴ The *cfy* protein could also be the Aurora A kinase that is required for proper centrosome separation with *aurora A* mutations or RNAi leading to monopolar mitotic spindles in *Drosophila* and *C. elegans*.^{30,34} Interestingly, in *Drosophila*, zygotic *aurora A* mutants develop through the syncytial blastoderm stage and display mitotic phenotypes only at later stages, perhaps due to a maternal component.²⁸ *cfy* mutants also progress through the early stages of development and only begin exhibiting mitotic phenotypes following neurulation.³⁶ Molecular identification of the *cfy* gene will provide additional insight into our understanding of how the regulation of centrosome assembly and the cell cycle is linked to the neural development.

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Disclosure Statement

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