Zebrafish cdc6 hypomorph mutation causes Meier-Gorlin syndrome-like phenotype

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Abstract

Cell Division Cycle 6 (Cdc6) is a component of pre-replicative complex (preRC) forming on DNA replication origins in eukaryotes. Recessive mutations in ORC1, ORC4, ORC6, CDT1 or CDC6 of the preRC in human cause Meier-Gorlin syndrome (MGS) that is characterized by impaired post-natal growth, short stature and microcephaly. However, vertebrate models of MGS have not been reported. Through N-ethyl-N-nitrosourea mutagenesis and Cas9 knockout, we generate several cdc6 mutant lines in zebrafish. Loss-of-function mutations of cdc6, as manifested by cdc6tsu4305 and cdc6tsu7cd mutants, lead to embryonic lethality due to cell cycle arrest at the S phase and extensive apoptosis. Embryos homozygous for a cdc6 hypomorphic mutation, cdc6tsu21cd, develop normally during embryogenesis. Later on, compared with their wild-type (WT) siblings, cdc6tsu21cd mutant fish show growth retardation, and their body weight and length in adulthood are greatly reduced, which resemble human MGS. Surprisingly, cdc6tsu21cd mutant fish become males with a short life and fail to mate with WT females, suggesting defective reproduction. Overexpression of Cdc6 mutant forms, which mimic human CDC6(T323R) mutation found in a MGS patient, in zebrafish cdc6tsu4305 mutant embryos partially represses cell death phenotype, suggesting that the human CDC6(T323R) mutation is a hypomorph. cdc6tsu21cd mutant fish will be useful to detect more tissue defects and develop medical treatment strategies for MGS patients.

Introduction

During DNA replication in eukaryotes, multiple replication origins are first recognized and bound during early G1 phase of the cell cycle by the origin recognition complex (ORC) consisting of ORC1-6 (1,2). Then, Cdc6, Cdt1 and the MCM2-7 helicase complex are sequentially recruited to each ORC-bound origin to form a pre-replicative complex (preRC) so that the origin is licensed for DNA replication. During the S phase, other factors are recruited to the preRCs to initiate DNA synthesis. It has been found that recessive mutations in ORC1, ORC4, ORC6, CDT1 or CDC6 of the preRC in human cause Meier-Gorlin syndrome (MGS) (3), also called ear patella short stature syndrome, which is characterized by impaired pre- and post-natal growth, short stature, microcephaly, microtia and absent or small patellae (2,4). A significant proportion of MGS patients also have abnormalities of genital and secondary sexual development (4). It remains unknown whether the reproduction and longevity of MGS patients are affected (2,4).

Cdc6 belongs to the AAA+ ATPase family and is essential for preRC formation by helping MCM proteins load onto replication origins. Cdc6 can also activate Cdk2 to promote S phase progression and G1 to S phase transition (5,6). In addition, Cdc6 has been found to inhibit apoptosome assembly and cell death by forming stable complexes with activated Apaf-1 molecules (7), to participate in spindle formation (8–10), and to regulate transcription of several cancer-related genes (11) and ribosomal DNA (12). Up-regulated expression of CDC6 has been found to
associate with the malignant progression of various tumors, and so CDC6 is a candidate prognostic marker and therapeutic target of several types of cancers (13–18). In the zebrafish, co-overexpression of cdc6 and c-myc in skin stimulates tumor-like transformation and genome instability (19). One MGS patient has been found to be homozygous for the missense mutation 968 C > G in the Cdc6 coding region, leading to the T323R substitution in the ATPase domain (3). The unavailability of animal models of Cdc6 mutation-caused MGS makes it difficult to investigate underlying cellular and molecular mechanisms. In addition, it remains unknown whether Cdc6 is essential for embryonic development in vertebrates.

In this study, we report several zebrafish mutant lines that carry different mutations in the cdc6 gene. Both cdc6

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which harbors a point mutation induced by N-ethyl-N-nitrosourea (ENU) mutagenesis, and cdc6

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with a curved trunk (Fig. 1A) and eventually died around 2–3 days post-fertilization (dpf). Abnormal embryos usually accounted for about 25% among siblings (see an example in Fig. 1B), which fits the Mendelian Law of Segregation. Thus, those abnormal embryos are zygotic mutants that are homozygous at a single mutant locus.

To identify the gene responsible for tsu4305 mutant phenotype, we performed positional cloning assay as described previously (20). Genetic mapping located the mutation site to a 3-Mb region harboring mpr4, jupa, cdc6, pimr93 and palm1a loci on chromosome 11 (Fig. 1C). Sequencing of coding regions of these genes identified a C > A point mutation in the ninth exon of cdc6. This nucleotide substitution would result in replacement of the nonpolar residue alanine by the negatively charged residue aspartic acid at position 406 of Cdc6 protein (Fig. 1D), which is defined as A406D. Zebrafish Cdc6 shares 60.7% homology of amino acid sequence with human CDC6, and in particular, known D-box, Cy-motif, AAA+ ATPase domain and winged helix-turn-helix DNA-binding domain (DBD) are highly conserved. However, the A406-containing region of Cdc6 is conserved across vertebrate species (Fig. 1D) with no defined function. The A406D substitution in tsu4305 mutants may change the properties and thus functions of Cdc6 protein.

PCR analysis of the cdc6 mutant region with two pairs of specific primers revealed that all abnormal embryos were homozygous for the mutant allele while normal embryos were either homozygous for the wild-type (WT) allele or heterozygous for WT and mutant alleles (exemplified in Fig. 1F), suggesting that cdc6 is the causative mutant gene. Whole-mount in situ hybridization showed that cdc6 transcripts were abundant with ubiquitous distribution from fertilization through gastrulation, and persisted at high levels in the central nervous system (CNS) at 24 hpf (Fig. 1G, upper panel). High levels of cdc6 expression in the CNS, a tissue with vigorous cell proliferation, may explain why cell death in cdc6

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mutant embryos happens first in the head at 22 hpf and became more prominent in the CNS than other tissues at 24 hpf (Fig. 1A). Cdc6 protein level was high before and at the gastrulation onset (6 hpf) and then declined (Fig. 1G, lower panel). In tsu4305 mutants, the cdc6 mRNA level appeared comparable to WT siblings (Fig. 1H and I). However, Cdc6 protein level in mutants was much higher than in WT siblings (Fig. 1J). Given that Cdc6 protein level fluctuates during cell cycle (21,22), higher levels of mutant Cdc6 protein suggest that many embryonic cells in mutant embryos are arrested at the phases during which Cdc6 normally accumulates, or that Cdc6(A406D) mutant protein is more stable.

tsu4305-encoded Cdc6(A406D) protein is non-functional

To investigate whether the tsu4305 phenotype is caused by loss or gain of function of Cdc6, we first performed rescue experiments. Over 90% of mutant embryos injected at one-cell stage with WT cdc6 mRNA did not show degenerative CNS at 24 hpf and had a straight trunk with death of some cells in the head at 48 hpf (Fig. 2A and B), suggesting a temporary rescue effect. In contrast, injection of tsu4305 mutant mRNA in WT or mutant embryos had no effect (data not shown). Next, we designed two antisense morpholinos to block expression of cdc6 mRNA in WT embryos (Fig. 2C). WT embryos injected with 20 ng of either MO phenocopied tsu4305 mutants (data not shown); however, tsu4305 heterozygous embryos injected with 10 ng of either MO showed similar phenotype (Fig. 2D). These results suggest that tsu4305 is most likely a null allele. Finally, we generated cdc6 mutants using CRISPR/Cas9 technology to target the third exon of the cdc6 locus. Consequently, we identified two mutant lines, cdc6

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(described later), which carried a 21-bp deletion in frame and homozygous mutant fish are smaller with infertility and a short life, which resemble human MGS. The cdc6

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that can be used as an animal model of MGS to identify tissue and organ defects in detail and develop medical treatment strategies for MGS patients.

Results

tsu4305 mutants are embryonic lethal and carry a point mutation in the cdc6 locus

During an ENU-mediated mutagenesis screen, we identified the tsu4305 line. Around 22-h post-fertilization (hpf), a proportion of embryos derived from crosses between heterozygotes looked darker in the head due to extensive cell death (Fig. 1A). These embryos thereafter exhibited increasing degenerative tissues with a curved trunk (Fig. 1A) and eventually died around 2–3 days post-fertilization (dpf). Abnormal embryos usually accounted for about 25% among siblings (see an example in Fig. 1B), which fits the Mendelian Law of Segregation. Thus, those abnormal embryos are zygotic mutants that are homozygous at a single mutant locus.

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Embryonic cells in tsu4305 are arrested at the S phase and undergo apoptosis

Given that Cdc6 is required for DNA replication in yeast and mammalian cells, we asked whether DNA replication in embryonic cells of zygotic tsu4305 mutant embryos is affected. To address this issue, we performed FACS-based cell cycle analysis using embryos at different stages, which were derived from crosses between tsu4305 heterozygous fish. Embryos at 20 hpf, at which there was no visible phenotype in mutants, were individually lysed and genotyped. We found that, compared with WT siblings, mutant embryos had more cells stopped at the S phase with less cells at the G2/M, S/G1, G0/G1, and G1 phases (Fig. 3A). At 22 and 24
When massive apoptotic cells in mutants appeared, many more cells in mutant embryos were arrested at the S phase with a decreased proportion of cells at the G0/G1 and G2/M phases (Fig. 3A). Consistent with the S-phase arrest, larger nuclei were observed in mutants (Fig. 3B). Cdk1 and Cdk2 can be activated by dephosphorylation at Tyr15, which promotes the S phase (23,24). We found that levels of Cdk phospho-Tyr15 were increased markedly in mutant embryos (Fig. 3C), which are

Figure 1. tsu4305 mutants are embryonic lethal due to a mutation of the cdc6 locus. (A) Morphology of mutants at indicated stages compared with WT siblings. (B) Percentage of mutant embryos at 24 hpf derived from a single family. n, total number of embryos. (C) The mutant gene is mapped to a region on chromosome 11. Polymorphic markers were shown with the recombinant rate in parentheses and candidate genes were indicated. (D) The point mutation in the 9th exon of the cdc6 locus results in a substitution of A406D in Cdc6 protein. Alignment of amino acid sequences in a region surrounding A406 of Cdc6 was illustrated on the top. The substituted residue was indicated by arrowheads. (E) Genotyping of embryos in a mutant family. Embryos derived from a single pair of tsu4305 heterozygous fish were morphologically divided at 24 hpf into normal and affected groups and genotyped using two pairs of specific primers (indicated on the top). h, w and m represent heterozygous, WT and mutant genotypes, respectively. (F) Expression of Cdc6 in WT embryos. cdc6 mRNA level in mutants is comparable to that in WT siblings as examined at 24 hpf by in situ hybridization (top panel) and protein levels were examined by Western blotting (bottom panel) at indicated stages. cdc6 mRNA level in mutants is comparable to that in WT siblings as examined at 24 hpf by in situ hybridization (top panel) and protein levels were examined by Western blotting (bottom panel) at indicated stages. cdc6 mRNA level in mutants is comparable to that in WT siblings as examined at 24 hpf by in situ hybridization (top panel) and protein levels were examined by Western blotting (bottom panel) at indicated stages. cdc6 mRNA level in mutants is comparable to that in WT siblings as examined at 24 hpf by in situ hybridization (top panel) and protein levels were examined by Western blotting (bottom panel) at indicated stages.
consistent with the increased levels of Cdc6 (Fig. 1J).

Furthermore, BrdU incorporation assay showed that much less amount of BrdU was incorporated into DNA in mutants (Fig. 3D), which supports the idea that the cell cycle is arrested. We wondered whether the S-phase arrest of tsu4305 mutant cells is accompanied by abnormal segregation of chromosomes. To address this question, we examined spindle formation and chromosome segregation in tsu4305 mutants by immunofluorescence with anti-α-tubulin antibody for spindles and DAPI for chromosomes. Results showed that tsu4305 mutants at 24 hpf had a much higher proportion of abnormal mitotic cells with disordered chromosomal arrangement at various phases (Fig. 3E). Interestingly, three genes, stag1, cnpF and aspm, which are involved in spindles formation and chromosomes segregation, were down-regulated in tsu4305 mutants (Fig. 3F). These results indicate that incomplete DNA replication due to deficiency of zygotic functional Cdc6 is associated with abnormal segregation of chromosomes.

Considering that Cdc6 also plays a role in prevention of apoptosis by blocking apoptosome assembly (7), we guess that cell

Figure 2. cdc6 morphants and knockout mutants phenocopy tsu4305 mutants. Rescue effect of cdc6 mRNA overexpression in tsu4305 mutants. One-cell stage embryos derived from tsu4305 heterozygote crosses were injected with different doses of cdc6 mRNA and then individually photographed followed by genotyping at 24 or 48 hpf. Only embryos homozygous for tsu4305 were chosen for categorizing into three types. (A) Morphology of injected tsu4305 mutant embryos in different categories. (B) Percentage of embryos in each category. (C) Effectiveness of cdc6 MOs. WT embryos at the one-cell stage were injected with 60 pg of the reporter pcd6-5’ UTR-GFP DNA in combination with different MOs and fluorescence was examined by microscopy at the shield stage. std MO, standard control MO. (D) Morphology of cdc6mutant heterozygous embryos injected with 10 ng of corresponding MO. The ratio of embryos with representative morphology was indicated. (E) Knockout of cdc6 by Cas9 technology. The cdc6mut allele was identified after targeting the 3rd exon of the cdc6 locus (top panel), which carried a 7-bp deletion so that a premature stop codon was introduced. The predicted mutant protein is truncated (bottom panel). Db, D-box; Cy, Cy-motif; DBD, DNA-binding domain; *, serine phosphorylation sites. (F) Morphology of cdc6mut homozygous mutants. WT, wild-type; MT, mutant. cdc6 mRNA (G) and protein levels (H) in cdc6mut mutants, which were examined at indicated stages by in situ hybridization and Western blotting. Note that lower amount of Cdc6 protein in mutants could be maternally supplied protein or translated from maternal cdc6 mRNA.
Figure 3. Mitotic defects in cdc6tsu4305 mutants. (A) DNA content analysis by flow cytometry. Embryos at 20 hpf were individually genotyped prior to flow cytometry and then grouped accordingly. Embryos at 22 and 24 hpf were identified by morphology. The percentages of cells at different phases were shown in lower panel based on three independent experiments. WT, homozygous WT genotype at 20 hpf or morphological WT at 22 and 24 hpf; MT, cdc6tsu4305 mutants. Statistical significance levels: **P < 0.01; ***P < 0.001; ns, non-significant (P > 95%). (B) Morphology of nuclei of retinal epithelia at 24 hpf. Nuclei were visualized by DAPI staining and observed by confocal microscopy (top panel). The average area of nuclei was calculated based on 50 cells (n) using Image J software. (C) Phospho-Cdk1/2 levels are increased in cdc6tsu4305 mutants (MT) at 24 hpf. The relevant proteins levels were examined by Western blotting. (D) Cell proliferation in cdc6tsu4305 mutants is impaired as revealed by BrdU incorporation. Top panel illustrated processes of embryo treatments in BrdU. Bottom panel showed immunofluorescence results following immunostaining of treated embryos with anti-BrdU antibody (green) and DAPI (blue). Abnormal segregation of chromosomes in cdc6tsu4305 mutants. Mitotic spindles were labeled by anti-α-tubulin antibody immunostaining (green) and chromosomes were stained by DAPI (blue). The showed mitotic cells (E) were chosen from the spinal cord at 24 hpf for confocal microscopy. The percentage of cells undergoing abnormal mitosis was shown in (F). W, wild-type embryos; M, mutant embryos. The total number of observed mitotic cells was 20 for each group. (G) Expression of three genes related to spindle function and chromosomal segregation is up-regulated in cdc6tsu4305 mutants at 22 and 24 hpf as analyzed by RT-PCR. Statistical significance levels (three independent experiments): *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant (P > 95%).
mRNA injection, injection of either cdc6 mutant mRNAs, amino acid glycine in yeast (Fig. 2E). We made the mutant forms human and the second arginine residue is replaced by the polar mutants. These two arginine residues are conserved from fly to which results from gradual loss of functional maternal Cdc6 phosphorylated at S139, a marker for DNA double-strand breaks (25), assay result showed massive DNA fragmentation in tsu4305 immediately N-terminal to A406 that is mutated to 406D in ATPase domain.

cdc6 mutant embryos can be rescued provisionally by overexpression of WT cdc6 mRNA into tsu4305 mutant embryos was unable to alleviate cell death (Fig. 5E). Interestingly, overexpression of human CDC6(193), which deleted the first 92 residues at the N-terminus including the D- and KEN-boxes and would lead to more stability of the protein (28,30), effectively blocked cell death in tsu4305 mutants. Western blotting result indeed showed that injection of human CDC6 mRNA into WT embryos produced undetectable level of the protein product while injection of CDC6(192) mRNA gave rise to massive protein product; mouse or Xenopus Cdc6 mRNA injection resulted in protein product at levels much lower than that from CDC6(192) mRNA injection (Fig. 5F). We then made the construct zCdc6(+hKEN) by inserting a KEN box containing peptide (GKKENGPPH) derived from human CDC6 into zebrafish Cdc6 and the construct zCdc6(+hCXDC6-N100) by replacing the N-terminal 113-residue portion of zebrafish Cdc6 with the N-terminal 100-residue fragment of human CDC6. Overexpression of either construct effectively rescued tsu4305 mutants (Fig. 5G), implying that the KEN box and other motifs in the N-terminal part of human CDC6 may not be responsible for protein instability in zebrafish embryos. It is likely that the molecular mechanisms regulating Cdc6 function in embryos may differ among species.

cdc6 mutant embryos undergo excess apoptosis. (A) Detection of active Caspase3. Embryos at 24 hpf were subjected to immunostaining with anti-active Caspase3 antibody and staining with DAPI and observed by confocal microscopy. All embryos were positioned with anterior to the left. (B) Detection of p-H2A.X in 24-hpf embryos by Western blotting. WT, wild-type siblings; MT, cdc6(265R) mutants.

cdc6 mutant fish become males with a smaller size and a shorter life

The mutant line cdc6(265R) originated by Cas9 gene targeting, carries a 21-bp deletion in frameshift of the CDC6 locus. The resulted mRNA is expected to produce a protein with deletion of seven residues (K77QRCAP150) in between the D-box and Cy-motif and the C104L substitution immediately adjacent to the Cy motif (Fig. 6A). The importance of this deleted region is not yet known. The cdc6(265R) mutant embryos grew normally and did not exhibit morphological defects before 15 dpf. However, at 30 dpf, mutant fish were smaller with a significantly reduced body length compared with their WT or heterozygous siblings (Fig. 6B). As measured at 100 dpf when fish are in young adulthood, the average body weight and length of

Figure 4. cdc6tsu4305 mutant embryos undergo excess apoptosis. (A) Detection of active Caspase3. Embryos at 24 hpf were subjected to immunostaining with anti-active Caspase3 antibody and staining with DAPI and observed by confocal microscopy. All embryos were positioned with anterior to the left. (C) Detection of p-H2A.X in 24-hpf embryos by Western blotting. WT, wild-type siblings; MT, cdc6(265R) mutants.

Since tsu4305 mutant embryos can be rescued provisionally by overexpression of WT cdc6 mRNA (Fig. 2A and B), they would be useful for verifying known or unknown functional domains of Cdc6. Then, we made deletional or mutational forms of cdc6 and tested their activity by overexpression in tsu4305 mutants.

The Walker A and the Walker B motifs in the AAA+ ATPase domain of Cdc6 protein are essential for loading McM complexes onto replication origins (26,27). We made two zebrafish cdc6 mutant mRNAs, cdc6(K224A) and cdc6(E294Q), which lead to K224A substitution in the Walker A motif and E294Q substitution in the Walker B motif respectively (Fig. 5A). Unlike WT cdc6 mRNA injection, injection of either cdc6(K224A) or cdc6(E294Q) mRNA into tsu4305 mutant embryos failed to prevent cell death as observed at 24 hpf (Fig. 5B), confirming the importance of the ATPase domain.

Zebrafish Cdc6 has two consecutive arginine residues immediately N-terminal to A406 that is mutated to 406D in tsu4305 mutants. These two arginine residues are conserved from fly to human and the second arginine residue is replaced by the polar amino acid glycine in yeast (Fig. 2E). We made the mutant forms Cdc6(R404A), Cdc6(R405A) and Cdc6(R404,405A), which changed arginine to the nonpolar residue alanine at the corresponding positions. Overexpression of mRNAs encoding these mutant forms of Cdc6 in tsu4305 mutant embryos was totally unable to inhibit cell death at 24 hpf (Fig. 5C). We therefore believe that R404 and R405 located outside the ATPase domain are absolutely required for normal function of Cdc6.

In the N-terminal region of Cdc6, the D-box is required for ubiquitination and degradation (28), and cyclin-binding (Cy) motif and several Cdk phosphorylation sites are implicated in interaction with Cdns and cytoplasmic translocation (29). We found that overexpression of cdc6(1104), which lacks the D-box, could rescue tsu4305 mutants as efficiently as did WT cdc6 (Fig. 5D), suggesting that Cdc6(1104) is functional in embryos. When compared with cdc6(1104), rescue effect of cdc6(1180), which deleted the D-box, Cy-motif and all known N-terminal phosphorylation sites, was slightly reduced, implying that interaction with Cdns is required for full activity of Cdc6. Like cdc6(K224A) (Fig. 5B), cdc6(A240) that deletes all of the N-terminal motifs as well as a part (including the Walker A motif) of the ATPase domain, completely lost the rescue activity in tsu4305 mutants (Fig. 5D). Generally, these data are in agreement with the previous findings on functions of Cdc6 N-terminal domains.

Next, we investigated functional conservation of Cdc6 from different vertebrate species. To our surprise, we observed that injection of human, mouse or Xenopus full-length Cdc6 mRNA into tsu4305 mutant embryos was unable to alleviate cell death (Fig. 5E). Interestingly, overexpression of human CDC6(193), which deleted the first 92 residues at the N-terminus including the D- and KEN-boxes and would lead to more stability of the protein (28,30), effectively blocked cell death in tsu4305 mutants. Western blotting result indeed showed that injection of human CDC6 mRNA into WT embryos produced undetectable level of the protein product while injection of CDC6(192) mRNA gave rise to massive protein product; mouse or Xenopus Cdc6 mRNA injection resulted in protein product at levels much lower than that from CDC6(192) mRNA injection (Fig. 5F). We then made the construct zCdc6(−hKEN) by inserting a KEN box containing peptide (GKKENGPPH) derived from human CDC6 into zebrafish Cdc6 and the construct zCdc6(−hCXDC6-N100) by replacing the N-terminal 113-residue portion of zebrafish Cdc6 with the N-terminal 100-residue fragment of human CDC6. Overexpression of either construct effectively rescued tsu4305 mutants (Fig. 5G), implying that the KEN box and other motifs in the N-terminal part of human CDC6 may not be responsible for protein instability in zebrafish embryos. It is likely that the molecular mechanisms regulating Cdc6 function in embryos may differ among species.
mutant fish were only 36.6 and 69.6% of the WT siblings (Fig. 6C), respectively. The body weight and length of heterozygous fish at 100 dpf were 88.7 and 99.6% of the WT siblings, respectively, which were not statistically significant. Apparently, the growth of \textit{cdc6}tsu21cd mutant fish is retarded, which resembles short stature characteristic of human MGS (2,4).

We extended our observation to mating behavior and longevity of \textit{cdc6}tsu21cd mutant adults. Most of the mutant fish looked slim, a typical feature of males, and the other had a slightly floated belly, an indication of potential females. When 12 young \textit{cdc6}tsu21cd mutant adults aging from 3- to 5-months old were individually mated to WT males or females, however, none of the mating pairs laid eggs, suggesting a failure of reproduction. It was observed that, when placed together with a WT male or female, the mutant fish did not move much and always tried to escape from the WT fish’s approach. Then, 5 mutant fish were anatomically analyzed. Surprisingly, all of the analyzed mutant fish had smaller testes with sperms (Fig. 6E–H), indicating that they were male.
males. It is believed that zebrafish larvae at 10–14 dpf all form a pair of juvenile ovaries as the default fate of gonad primordia; and these juvenile ovaries will be transformed into testes if exposed to a stress such as less food, insufficient oxygen and inappropriate temperature (31). Therefore, we hypothesize that the \textit{cdc6\textsuperscript{tsu21cd}} mutant larvae are most likely to become males because of poor health condition. But, it remains elusive why the mutant males fail to mate with WT female.

We raised \textit{cdc6\textsuperscript{tsu21cd}} mutant fish, heterozygous and WT siblings separately but in the same conditions after genotyped at one month old, which could avoid competition. Observation of two batches of siblings indicated that none of \textit{cdc6\textsuperscript{tsu21cd}} mutant fish was alive beyond 8 months, while WT and heterozygous siblings were still alive after 14 months (Table 1). The zebrafish normally has an average life span of 36–42 months depending on strains (32). Apparently, \textit{cdc6\textsuperscript{tsu21cd}} mutant fish are short-lived. We observed that the \textit{cdc6\textsuperscript{tsu21cd}} mutant fish had an abnormal head with misshapen mouth (Fig. 6C), swam slowly and did not eat food actively (Supplementary Material, Movie S1). In fact, the \textit{cdc6\textsuperscript{tsu21cd}} mutant adults showed many abnormal tissues including the spine, swimming bladder, intestine, livers and so on (Fig. 6F, I and J). In general, these defects all might cause health problems and ultimately lead to a shorter life. The major causes of the reduced lifespan of the mutant fish need to be investigated in depth in the future.

Figure 6. Growth retardation and abnormal tissues of \textit{cdc6\textsuperscript{tsu21cd}} mutants. (A) Illustration of \textit{cdc6\textsuperscript{tsu21cd}} mutant allele and protein. The deleted nucleotides and residues were represented by dashes and substitutions were labeled in green letters. Db, D-box; Cy, Cy-motif; DBD, DNA-binding domain. (B) Morphology (left) and body length (right) of larva at 30 dpf. Larva derived from \textit{cdc6\textsuperscript{tsu21cd}} heterozygous fish matings were genotyped using tail fin DNA. Morphology (C), weight and body length (D) of fish at 100 dpf. Embryos derived from \textit{cdc6\textsuperscript{tsu21cd}} heterozygous fish matings were raised to 30 dpf when genotyping was performed using tail fin DNA and then continued growing to 100 dpf. n, number of measured larva or adults. Statistical significance levels: ***P < 0.001; ns, non-significant (P > 95%). (E) Visualization of testes in adult fish. (F) Cross section of adult fish after hematoxylin and eosin (H&E) staining. Testes (T) were indicated by red arrows. Green arrow-indicated tissues were: S, spine; K, kidney; Sp, swimming bladder; L, liver; Ip, intestinal loops. (G) The testes boxed in (F) were observed at high power. (H) Enlarged spine (S) shown in (F). (I) Enlarged intestinal loop shown in the upper right in (F).
Zebrafish cdc6tsu21cd and human CDC6(968C > G)(T323R) are hypomorphic alleles

In comparison with cdc6tsu4305 and cdc6tsu7d mutants that are embryonic lethal, cdc6tsu21cd mutants are normal during embryonic and larval stages, suggesting that cdc6tsu21cd-encoded protein retains certain functional activity. To test this possibility, we injected synthetic cdc6tsu21cd mRNA (Fig. 7A) into one-cell stage cdc6tsu4305 embryos and observed morphology and performed TUNEL assay at 24 hpf, which were followed by genotyping. Results showed efficient prevention of cell death in cdc6tsu4305 mutant embryos injected with cdc6tsu21cd mRNA (Fig. 7A and B), suggesting that cdc6tsu21cd-encoded protein has considerably high levels of functional activity.

A human CDC6(968C > G) homozygous patient develops MGS (3). This mutation is expected to encode CDC6(T323R) mutant protein. As shown above, overexpression of human WT CDC6 mRNA failed to rescue cdc6tsu4305 mutant embryos, but, overexpression of human CDC6(A92) mRNA coding for N-terminal truncated form had a good rescue effect (Fig. 5E). Then, we made human CDC6(T323R-A92) mRNA (Fig. 7A) to perform rescue experiments. As shown in Figure 7B and C, cell death in injected cdc6tsu4305 mutants was not as severe as in uninjected mutants, and the number of apoptotic cells in the head and spinal cord of injected mutants appeared slightly reduced. The threonine residue at position 323 of human CDC6 has the counterpart at position 332 of zebrafish Cdc6. So, we made zebrafish cdc6(T323R) mRNA to do rescue experiments and found a weak rescue effect in cdc6tsu4305 mutants (Fig. 7B and C), which was similar to injection of human CDC6(T323R-A92) mRNA. These results imply that T323R substitution of human CDC6 may lead to partial loss of function.

We noted that the expression of the apoptosis-related genes tp53 and bbc3 was highly up-regulated in cdc6tsu4305 mutants (Fig. 7D) so that they could be used as markers to quantitatively verify the rescue effect. Overexpression of cdc6(A406D) in cdc6tsu4305 mutants had no effect on tp53 and bbc3 expression and so this injection was used as control. When compared with cdc6(A406D) injection, zebrafish cdc6 or cdc6tsu21cd or human CDC6(A92) mRNA injection significantly repressed tp53 and bbc3 expression in cdc6tsu4305 mutants at 24 hpf, and zebrafish cdc6(T323R) or human CDC6 or Cdc6(T323R-A92) mRNA injection also compromised upregulation of tp53 and bbc3 expression in cdc6tsu4305 mutants though to a lesser extent. Therefore, we believe that both zebrafish cdc6tsu21cd and human CDC6(T323R) are hypomorphic mutant alleles. This explains why the human patient carrying homozygous CDC6(T323R) mutation was alive after birth.

Discussion

A human patient with MGS has been found to be homozygous for CDC6(T323R) mutation. However, genetic mutants of Cdc6 in vertebrates have not been generated. By forward and reverse genetic approaches, we generate two loss-of-function mutant lines for cdc6 in zebrafish, cdc6tsu4305 and cdc6tsu7d. We show that zygotic deficiency of functional Cdc6 causes embryonic lethality due to cell cycle arrest and extensive apoptosis. In zebrafish, maternal cdc6 transcripts are present in mature oocytes. These maternal messages would be sufficient to allow normal development of embryos from fertilization through somitogenesis. When maternally supplied Cdc6 gets less and less, mitosis of embryonic cells is halted mostly at the S phase, which ultimately leads to death of cells. The lethality of embryos without Cdc6 makes it difficult to study Cdc6 implication in germ cell formation and oocyte maturation in zebrafish. It is possible to study functions of maternal Cdc6 through conditional knockout.

The cdc6tsu4305 line harbors a single nucleotide mutation in the coding region, which would produce the mutant protein Cdc6(A406D) (Fig. 1D). However, the alanine residue at 406 is conserved in Cdc6 homologues across vertebrate species, and is changed to the uncharged polar residue serine in yeast and the nonpolar residue valine in fly (Fig. 1E). We note that two consecutive positively charged arginine residues precede A406 in Cdc6. The A406D substitution in tsu4305 mutants may change the properties and thus functions of Cdc6 protein. As one piece of evidence, Cdc6 protein levels in mutant embryos are higher than in their WT siblings (Fig. 1F), suggesting that Cdc6(A406D) mutant protein is more stable though it is non-functional. In addition, overexpression of Cdc6(R404A) or Cdc6(R405A) fails to rescue cdc6tsu4305 mutant phenotype, indicating the importance of these residues for Cdc6 function. Based on the extrapolated 3-D structure of human CDC6 (33), RRA-containing region (R395KALDVCRRAIEIV345), which is conserved in zebrafish Cdc6, forms the 13th α-helix, which is close to the Mg-ADP binding pocket. Therefore, we speculate that Cdc6(A406D) may lose ATPase activity.

Our mutant line cdc6tsu21cd carries a hypomorphic mutant allele that encodes a Cdc6 mutant protein with slightly reduced activity. cdc6tsu21cd fish show growth retardation and shorter body length, which are similar to human MGS caused by mutations of preRC complex components (2–4). We demonstrate that the CDC6(T323R) mutation leading to MGS could be hypomorphic as well (Fig. 7). The cdc6tsu21cd mutant fish have many abnormal tissues such as the spine and swimming bladder, but fins and pigmentation appear normal (Fig. 6B and C), which suggests that different tissues are disturbed differentially. Given that cdc6tsu21cd fish develop MGS-like phenotype, this zebrafish mutant line could be used to further study how different tissues are affected and to develop medical treatment and care procedures for alleviating the defects.

Materials and Methods

Zebrafish strains, mutagenesis and mapping

Zebrafish Tuebingen (Tu) and India strains were used in this study with ethical approval from the Animal Care and Use Committee of Tsinghua University. Embryos were raised in Holtfreter’s solution at 28.5°C.

Chemical mutagenesis was performed as previously described (34,35). Briefly, WT Tu males aged 4–8 months were soaked into solution containing 3 mM ENU three times in one week and then crossed with WT India females to transmit induced mutations to next generation. Gene mapping was performed as previously described with modifications (20).

For Cas9-mediated gene targeting, gRNA target sequence (5’-GGACCCTGAACGGTTAGGAGG-3’) of zebrafish cdc6 was selected from the website (http://chopchop.cbu.uib.no/; data last accessed August 3, 2017). Synthetic Cas9 mRNA and gRNA were co-injected into Tu WT embryos at the one-cell stage. The founder fish (F0) and F1 fish carrying mutations were identified by digestion of T7 Endonuclease I that recognizes and cleaves non-perfectly matched DNA. Two mutant lines of cdc6, i.e. cdc6tsu21cd and cdc6tsu4305 were identified and maintained for analyses.

Genotyping

Two pairs of primers were used to identify WT cdc6 and cdc6tsu4305 mutant alleles by PCR. The sequences of primers

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were: 5′-CTGATATGTTACCAATAATGCTT-3′ (f1) and 5′-GCTTGGCTCTCCCCTCTCT-3′ (f2) for mutant allele: 5′-GCTTGGCTCTCCCCTCTCT-3′; and reverse primer for both alleles: 5′-AGGAAACAGTCGCGTAACAG-3′.

Constructs, morpholinos and microinjection
The full-length coding sequences of zebrafish cdc6 and human CDC6 were separately cloned into pCS2(+) vector with a Flag tag...
at N-terminal for in vitro synthesis of mRNAs. The mutant forms of zebrafish cdc6, including cdc6(A406D), cdc6(R224A), cdc6(E294Q), cdc6(R404A), cdc6(R405A), cdc6(R404,405A) and cdc6(T332R), were made by site-directed mutation on cdc6 containing vector. Three cdc6 truncated mutants, cdc6(Δ104), cdc6(Δ180) and cdc6(Δ240), were amplified from WT cdc6 and subcloned into pCS2(+) vector. The coding sequence of cdc6mut21cd was amplified from cdc6mut21cd mutant embryos and cloned into pCS2(+) vector. To validate the efficiency of two cdc6 morpohinos, a fragment containing 5’ untranslated region (5’- UTR) and 5’ part of coding sequence of cdc6 was amplified with the forward primer (5’- CGGAATTCTTGAGACGAGAATTACA-3’) and the reverse primer (5’- TCCCGCGAGGACGTGCTGGGATT-3’) and fused to the coding sequence of egfp, which were together inserted into pEGFP-N3 vector.

Capped mRNAs were synthesized using Sp6 mMessage mMachine Kit (Ambion) and purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Two morpholinos, cdc6-MO1 (5’-AAAGCTGTAGTTTCTCTGCTCCTGA-3’) and cdc6-MO2 (5’- GAGTGCTGGGACTTATGTTA-3’) were designed to inhibit Cdc6 translation, which were synthesized by Gene Tools, LLC. And std MO (5’-CTTATAGATGGCAGTGGCTC-3’) was used as a control. Morpholinos and mRNAs were individually injected into the yolk of one-cell stage embryos with the typical MPPI-2 quantitative injection equipment at indicated doses and collected for analysis at later stages.

**Whole-mount in situ hybridization and real-time quantitative RT-PCR**

A fragment containing 3’-UTR and 3’-part of coding sequence of cdc6 was amplified with the forward primer (5’- AAGATCAACTACACAGACTCACAGGTA-3’) and the reverse primer (5’- TATACTAGCTTAC TATAGGGGTATAAGGAGCAATGTCAAC-3’) and used for synthesizing Digoxigenin-labeled antisense RNA probe. Whole-mount in situ hybridization was performed using the commonly used protocol.

Total RNAs from embryos were used for real-time quantitative PCR. The primer sequences for analyzed genes were: for cdc6, 5’- GAGCCGAGATTGGGAGAA-3’ (forward) and 5’- AAAG TTGAGCAGTGGGAGAC-3’ (reverse); for β-actin, 5’- ATGGATGATGAAATTGGCCGAC-3’ (forward) and 5’- ACCATCACAGACGTCCA-3’ (reverse); and for β-tubulin, 5’- TGGCGCTTCTCCCTTCG-3’ (forward) and 5’- GG TCTGGCTTCTCCCTTCG-3’ (reverse); for cgf, 5’- GAGGGTCAATGAAGGAGAT-3’ (forward) and 5’- CTCGCAGGACTGAAAG-3’ (reverse); for cdc6, 5’- AGTGGGATGAAATTGGCCGAC-3’ (forward) and 5’- CTGCAATGGGAGGAGAT-3’ (reverse).

**Cell cycle analysis**

The cell cycle analysis was performed as before (36). Briefly, embryos were dechorionated at desired stages by pronase treatment and deyolked by pipetting with a 200-μl tip. For preparing single-cell suspension, embryos were incubated in 1 ml of 0.25% trypsin at 28 °C for 30 min with triturating repetitively using a 1-ml tip followed by adding 0.5 ml DMEM containing 10% fetal bovine serum to stop the activity of trypsin. The homogenates were passed through a 40-μm mesh filter (BD, no. 352235) to get single-cell suspension. The cells were collected by centrifugation at 3000 rpm for 3 min and resuspended with 0.25 ml of PBS followed by fixation with 0.75 ml of cold ethanol at 4 °C overnight. The fixed cells were precipitated and resuspended with 500 μl of propidium iodide solution (0.1 mg/ml propidium iodide, 0.1% sodium citrate, 100 μg/ml RNase A, and 0.0002% Triton X-100) followed by analysis using the BD FACS Aria II flow cytometer. For tsu4305 mutants and WT siblings at 22 and 24 hpf, 15 embryos were used for each group. For embryos at 20 hpf, the embryos were digested individually into single-cell suspension and genotyping was performed before further analysis. Each group contained two to three embryos.

**Western blot**

For preparing antibody against zebrafish Cdc6, the full-length coding sequence of cdc6 was cloned into the expression vector pET-15b. The recombinant plasmid was transformed into Escherichia coli BL21 cells and expression was induced by adding IPTG at 1 mM for 6 h at 16 °C. The recombinant protein was purified with Ni-affinity chromatography and injected into rabbit at 500 μg per time as antigen. After four times of immu- nization, the rabbits were killed to collect blood. Serums were gathered through deposition and centrifugation of blood. The antibody against zebrafish Cdc6 was purified by antigen coupled affinity chromatography. The antibody was dialyzed, aliquotted and stored at −80 °C.

Embryos were dechorionated at desired stages and deyolked by pipetting with a 200-μl tip. Cells were collected by centrifugation at 3,000 rpm for 3 min and lysed in TNE buffer for 3 min at 4 °C followed by centrifugation at 12,000 rpm for 10 min. The supernatant was added SDS loading buffer and boiled for 10 min. The used primary antibodies were: anti-actin (Santa Cruz, I-19, 1: 1000), anti-Cdk1 (Santa Cruz, sc-54, 1: 1000), anti-p-Cdk1 (CST, 9111, 1: 1000), anti-Cdk2 (Millipore, 05-596, 1: 1000), anti-Flag (MBL, M185-L, 1: 1000), anti-γ-H2A.X (CST, no. 9718, 1: 1000), and anti-zebrafish Cdc6 (home-made, 1: 5000). The used secondary antibodies were HRP-conjugated anti-mouse (GE, NA931-1ML, 1: 5000), HRP-conjugated anti-rabbit (GE, NA934-1ML, 1: 5000) or HRP-conjugated anti-goat (Jackson ImmunoResearch, 1: 5000).

**TUNEL, BrdU incorporation and immunostaining assays**

For detecting apoptosis, TUNEL assay was performed using ApopTag Red In Situ Apoptosis Detection Kit (Millipore, S7165) according to the manufacturer’s instruction. For BrdU incorporation assays, embryos at 23 hpf were transferred to Holtfreter’s solution containing 10 mM BrdU and incubated for 20 min on ice, and then dispersed to fresh Holtfreter’s solution and incubated at 30 °C for 20 min or 1 h, followed by fixation in 4% polyformaldehyde. Other procedures for BrdU incorporation and immunostaining assays were similar unless other stated. Briefly, embryos fixed in 4% polyformaldehyde were dehydrated with methanol and stored at −20 °C overnight, followed by dehydration in PBST (1XPBS, 0.5% Triton X-100). The embryos were then permeabilized in acetone for 7 min at −20 °C and washed three times with PBST. For BrdU immunostaining, embryos were treated in 2M HCl for 1 h at room temperature followed by washing with PBST. Next, embryos were incubated in the block solution (1% BSA, 10% serum in 0.5% PBST) for 1 h at room temperature, and transferred to the block solution containing corresponding primary antibody for incubation overnight at 4 °C. After rinsed four times for 15 min each in PBST, the embryos were incubated in the block solution containing a secondary antibody for 2 h at room temperature, followed by washing times four
Table 1. Survival rate of cdc6<sup>mut2ts</sup> mutants<sup>a</sup>

| Batches | Age (months) | Number of fish (survival rate)<sup>b</sup> |
|---------|--------------|------------------------------------------|
|         |              | +/+ | +/-- | --/-- |
| 1       | 1            | 6   | 15   | 6   |
| 1       | 6 (100%)     | 14  | (93.3%) | 6 (100%) |
| 2       | 6 (100%)     | 14  | (93.3%) | 4 (66.7%) |
| 3       | 6 (100%)     | 14  | (93.3%) | 0 (0%) |
| 7       | 13 (86.7%)   | 15  | (100%) | 13 (86.7%) |
| 5       | 13 (86.7%)   | 15  | (100%) | 9 (60%) |
| 7       | 13 (86.7%)   | 14  | (93.3%) | 1 (6.7%) |
| 8       | 13 (86.7%)<sup>c</sup> | 14 | (93.3%)<sup>c</sup> | 0 (0%) |

<sup>a</sup>Embryos derived from a single cdc6<sup>mut2ts</sup> heterozygous fish cross were raised to 30 dpf and then genotyped using tail fin DNA. The genotyped fish continued growing up.

<sup>b</sup>The survival rate was calculated based on the number of fish at 1-month old.

<sup>c</sup>These WT and heterozygous fish are still alive up to now (over 14 months).

...for 15 min each in PBST. The used antibodies were: primary anti-BrdU (Santa Cruz, sc-32323, 1: 100), anti-active caspase3 (BD Biosciences, 559565, 1: 500), primary anti-α-tubulin antibodies (Sigma, T5168, 1: 100) and secondary antibodies conjugated with DyLight Fluorescent Dyes (488/549) (Jackson ImmunoResearch, 1: 1:100) and secondary antibodies conjugated with secondary antibodies conjugated with p27 protein undergoes C-terminal phosphorylation. J. Biol. Chem., 287, 6275–6283.

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Statistical analysis

Data averaged from multiple samples were presented as mean ± standard deviation (mean ± SD). Significance of difference was analyzed by Student’s t-test.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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