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Loss-of-function of zebrafish *cdt1* causes retarded body growth and underdeveloped gonads resembling human Meier-Gorlin syndrome

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The cell cycle consists of four distinct phases: G0/G1, S (DNA synthesis), G2, and M (mitosis). The G1 to S transition is typified by an accumulation of 4',6-diamidino-2-phenylindole (DAPI) signal that indicates the rapid DNA synthesis initiated at special sites on DNA, generally called the *Ori* (origin of replication) (Alfa et al., 1989). The cell division cycle 10 (Cdc10)-dependent transcript 1 (Cdt1) is bestowed the term "replication origin licensing factor" for its role in warranting replication once (and only once) per round of the eukaryotic cell cycle, during the S phase (Pozo and Cook, 2017). In a normal cell cycle, Cdt1 is present only in the G1 and S entry phases, whereas Geminin, a protein that targets Cdt1 for S-phase-dependent proteolysis, is present in the S and G2 phases. Failure of this gatekeeping task as observed in *cdt1* overexpression, for example in yeast, leads to inappropriate origin firing (also termed re-replication (Vaziri et al., 2003)) and eventually DNA damage checkpoint activation (Kanellou et al., 2020).

Cdt1 is a crucial cell-cycle determinant, and was first cloned in fission yeast via an immunoprecipitation-polymerase chain reaction (PCR) strategy. The goal was to search for a novel target sequence that interacts with Cdc10, a transcription factor belonging to a conserved DNA-binding complex (Hofmann and Beach, 1994). Due to its high conservation across eukaryotic kingdoms (Pozo and Cook, 2017), *cdt1* homologues of *Drosophila* (Whittaker et al., 2000), X*enopus* (Maiorano et al., 2000), and *Schizosaccharomyces pombe* (Nishitani et al., 2000) were successively cloned via homology or functional approaches. By employing its cycling nature during the cell cycle, the human homologue was eventually obtained through interaction with GEMININ (Wohlschlegel et al., 2000). While most functions were elucidated via experiments in lower organisms, especially in yeasts, the importance and significance of CDT1 were subsequently confirmed and extended to cell-cycle-related human diseases; this was made possible by the completion of the human genome project and the availability of large patient datasets (de Munnik et al., 2015). Specifically, mutations in genes of the pre-replication complex, including CDT1, have been detected in approximately 67%–78% of patients with Meier-Gorlin syndrome (MGS), a rare autosomal recessive primordial dwarfism disorder which is characterized by impaired post-natal growth, short stature, and microcephaly (de Munnik et al., 2015). Using the model organism zebrafish, researchers found that overexpression of Cdc6 mutant forms mimicked the human CDC6 (T323R) mutation found in an MGS patient, raising the exciting possibility of using zebrafish mutants as animal models of MGS to identify tissue and organ defects in detail and develop medical treatment strategies for MGS patients (Yao et al., 2017). The recent discovery of gene compensation response (GCR) in the small teleost further supports the potential contributions of zebrafish genetics to human medicine (Ma et al., 2019).

In this study, we first analyzed the full-length zebrafish Cdt1 protein sequence decoded from a *cdt1* transcript (XP_695164.3) deposited in the National Center for Biotechnology Information (NCBI) database. Protein domain prediction using the Uniprot program

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revealed that the C-terminus contains well characterized functional domains, including the minichromosome maintenance protein (MCM)-binding domain (544–669 aa) and Geminin-binding domain (303–487 aa). These are responsible for preventing untimely MCM loading onto chromatin and origin licensing, respectively, during the cell cycle (Pozo and Cook, 2017); and they support the conserved role of Cdt1 in DNA replication (Fig. 1a). Interestingly, no known domain was returned for the N-terminal sequence (1–301 aa). Instead,

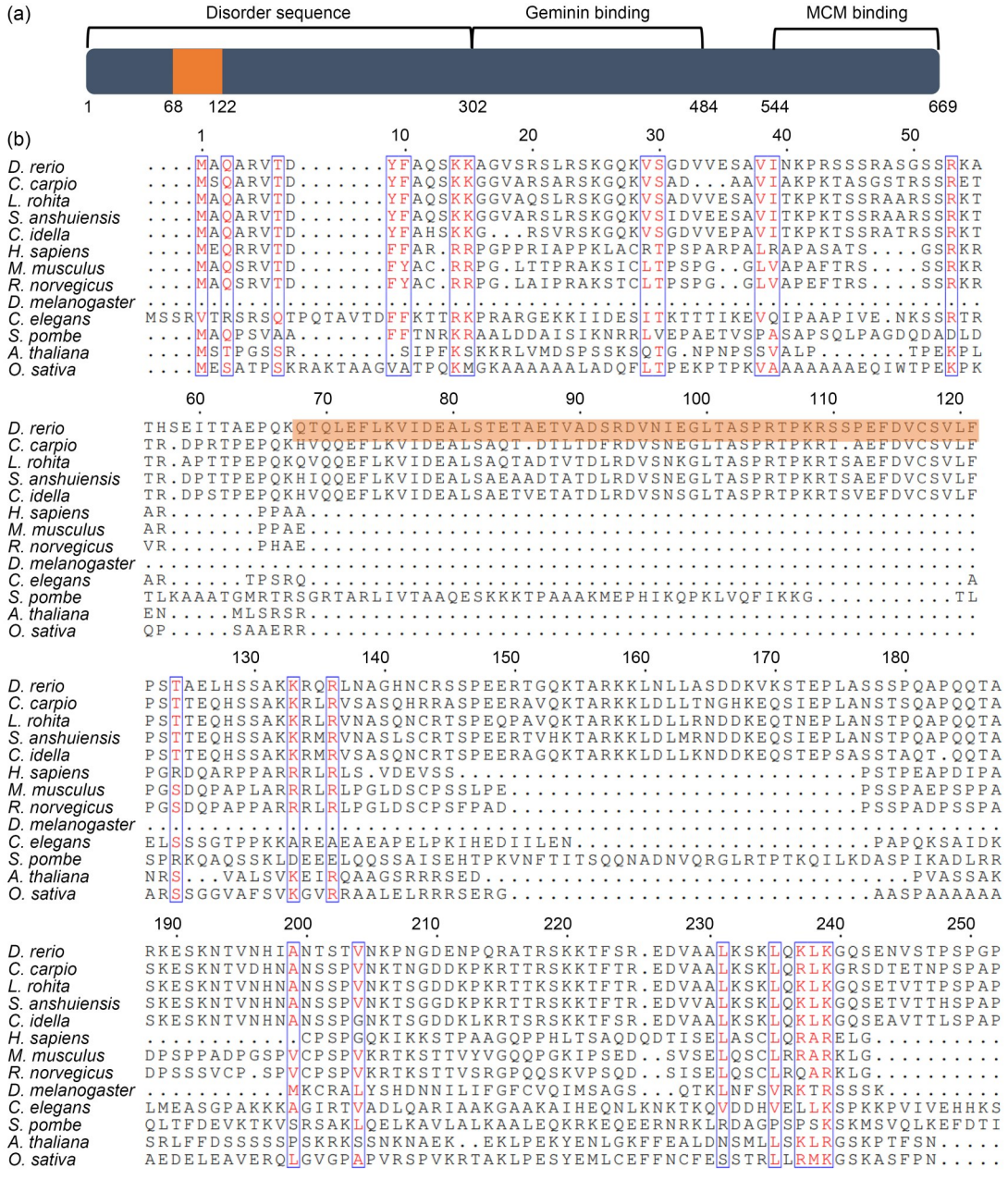

Fig. 1 A unique N-terminal found in zebrafish cell division cycle 10 (Cdc10)-dependent transcript 1 (Cdt1), specific to bony fishes. (a) Functional-domain prediction of zebrafish Cdt1 protein. The grey box represents Cdt1 protein; numbers depict amino acids. The 53 unique amino acids in the N-terminal domain are boxed in orange. (b) Multiple alignment of Cdt1 protein sequences obtained from thirteen organisms including: zebrafish (*Danio rerio*), four species in the Cypriniformes order (*Cyprinus carpio*, *Labeo rohita*, *Sinocyclocheilus anshuiensis*, and *Ctenopharyngodon idella*), human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), fruitfly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*), fission yeast (*Schizosaccharomyces pombe*), thale cress (*Arabidopsis thaliana*), and rice (*Oryza sativa*), at N-termini. Red text: similarity within a group; Blue box: similarity across groups; Dots: gaps.

we primarily found disordered residues (Fig. 1a). Upon multiple sequence alignment, we found that while the full length (1–678 aa) and C-terminal (302–678 aa) of zebrafish Cdt1 shared high homologies, for example, with their mouse CDT1 counterparts, with approximately 45.6% and 59.6%, respectively, alignment of the N-terminal (with 1–301 aa of zebrafish Cdt1 as the reference) yielded an identity value of only 19.4% with low significant *E*-value (Table 1), suggesting a unique N-terminus.

Table 1 Homologies of cell division cycle 10 (Cdc10)-dependent transcript 1 (Cdt1) sequences from 52 species at full length, N-terminal, and C-terminal.

Classification	Name	Identity of full length (%)	Identity of N-terminal (%)	Identity of C-terminal (%)
Mammalias	*Homo sapiens*	46.5	20.4	58.8
	Macaca mulatta	45.9	16.9	59.8
	Mus musculus	45.6	19.4	59.6
	Sus scrofa	46.4	22.1	60.0
	Canis lupus familiaris	43.7	17.9	60.5
	Myotis lucifugus	48.0	23.5	58.0
	Microcebus murinus	38.5	14.2	54.8
	Oryctolagus cuniculus	41.6	13.7	54.0
	Ochotona princeps	46.1	17.9	60.0
	Felis catus	46.7	20.6	60.1
	Erinaceus europaeus	44.0	15.6	62.5
	Sarcophilus harrisii	43.9	16.6	67.0
	Ornithorhynchus anatinus	47.7	20.5	60.0
	Equus caballus	47.0	21.4	59.6
	Dasypus novemcinctus	45.2	17.0	56.7
	Echinops telfairi	44.3	19.0	60.2
	Loxodonta africana	45.3	21.6	59.0
	Rattus norvegicus	45.0	19.6	61.8
Aves	*Anas platyrhynchos*	47.5	19.4	62.6
	Gallus gallus	45.1	18.3	61.6
	Meleagris gallopavo	49.6	17.2	59.6
	Taeniopygia guttata	42.3	19.7	68.2
Reptilia	*Chrysemys picta bellii*	45.3	20.5	61.6
	Thamnophis elegans	44.5	18.9	60.6
	Crotalus tigris	41.9	17.4	63.3
	Anolis carolinensis	47.1	19.7	64.0
	Gavialis gangeticus	53.3	24.5	67.8
Amphibias	*Xenopus tropicalis*	48.5	20.1	68.4
	Xenopus laevis	48.2	20.5	66.5
	Xenopus laevis	48.1	19.8	73.3
Osteichthyes	*Lepisosteus oculatus*	54.3	29.5	65.4
	Oncorhynchus mykiss	54.4	26.5	77.3
	Oncorhynchus mykiss	54.6	21.3	89.6
	Cyprinus carpio	79.4	72.6	89.6
	Cyprinus carpio	82.1	78.8	64.9
	Labeo rohita	91.9	71.6	66.5
	Sinocyclocheilus anshuiensis	90.9	70.3	65.3
	Ctenopharyngodon idella	90.9	70.3	65.5
Chondrichthyes	*Rhincodon typus*	43.4	18.3	62.6
	Callorhinchus milii	42.2	17.5	70.7

To be continued

Table 1 (continued)

Classification	Name	Identity of full length (%)	Identity of N-terminal (%)	Identity of C-terminal (%)
Cyclostomata	*Petromyzon marinus*	32.6	15.6	42.8
Ascidiacea	*Ciona intestinalis*	30.1	13.6	45.2
Leptocardii	*Branchiostoma belcheri*	40.2	20.3	45.2
	Branchiostoma floridae	30.2	16.9	44.1
Echinodermata	*Asterias rubens*	30.2	13.1	45.5
	Patiria miniata	31.3	14.6	46.5
	Acanthaster planci	31.4	14.2	42.6
	Strongylocentrotus purpuratus	29.4	15.8	44.1
	Anneissia japonica	31.0	15.2	47.3
Enteropneusta	*Saccoglossus kowalevskii*	32.4	15.6	47.3
Ascomycotina	*Schizosaccharomyces pombe*	14.2	16.9	14.5
Nematoda	*Caenorhabditis elegans*	14.7	14.0	17.8
Arthropoda	*Drosophila melanogaster*	24.6	12.8	35.6
Angiospermae	*Arabidopsis thaliana*	13.6	17.9	12.2
	Arabidopsis thaliana	12.6	24.3	12.1

To further explore the distinctiveness of this sequence, we constructed a phylogenetic tree involving 56 sequences from 52 species, encompassing dicot, protozoan, invertebrate, and vertebrate phyla. We then used MEGA7 to explore the evolutionary distance between Cdt1/CDT1 in these species, including seven species from the bony fish clade (Fig. 2). While the full-length protein was fully consistent with the classic classification based on anatomic characteristics, phylogenetic analysis of the Cdt1/CDT1 N-termini from these 52 species failed to yield a proper evolutionary relationship, mainly due to the divergent sequences from different phyla (Table 1). Strikingly, aligning the N-termini of Cdt1/CDT1 revealed a 53-aa insertion (69–121 aa in zebrafish Cdt1) which is only found in the four species in the Cypriniformes order. It is not found in other species such as human, mouse, rat, fly, worm, rice, *Arabidopsis*, or yeast (Fig. 1b), suggesting a unique origin of this sequence retained by this clade during evolution.

While quantitative real-time PCR (qPCR) (Fig. 3a), whole-mount in situ hybridization (WISH) (Fig. 3b), and western blot (Fig. 3c) showed that *cdt1*/Cdt1 at both the transcript and protein levels were maternal in nature and zygotic expression was maintained at low and ubiquitous levels, RNA sequencing (RNA-seq) data revealed that the *cdt1* was particularly enriched in adult testis and ovary tissues (Fig. 4d, lower panel), suggesting important roles in zebrafish reproductive organs. In contrast, the *cdt1* transcript was present at relatively low levels in other organs/tissues (transcripts per million (TPM)<2.5) (Fig. 4d, lower panel), indicating limited cell proliferation in these organs/tissues in adult fish.

To explore the function of *cdt1* in zebrafish, we designed a guide RNA (gRNA)-targeting zebrafish *cdt1* exon 2. We then generated a mutant allele ($cdt1^{zju1/zju1}$) of 4-bp deletion in exon 2, resulting in an early stop codon in exon 3 (Fig. 5a). This gave rise to a truncated and presumably unstable protein, leading to a null mutant (Fig. 3c). The protein was undetected in 3-day post fertilization (dpf) mutant embryos. Next, we sought to investigate the developmental status of the three basic germ layers during the early embryonic stage of the $cdt1^{zju1/zju1}$ mutant, by systematically scoring them with specific markers via WISH. No noticeable differences were detected in ectoderm-derived cranioskeletons (by Alcine blue staining), mesoderm-derived muscles (by α-tropomyosin, (*tpma*)), or endoderm-derived liver (by *fabp10a*), intestine (by *fabp2*), or exocrine pancreas (by *trypsin*) (Fig. 5b). We proceeded to raise the embryos laid by both heterozygous parents and traced $cdt1^{zju1/zju1}$ mutant lethality, if any. We found that while full survival was exhibited by 25% of the population up to 15 dpf, the $cdt1^{zju1/zju1}$ mutant ratio was drastically reduced at 1 month post fertilization (mpf), with only a few surviving homozygous fish by 2 and 3 mpf (sexual maturity of zebrafish occurs around 3 mpf) (Fig. 5c). Notably, we found that the surviving $cdt1^{zju1/zju1}$ mutants experienced a growth disorder, in which their body size was less than 50% of that of the wild type (WT) (Fig. 5d). This observation reminded us of human MGS patients, who characteristically carry mutations in multiple

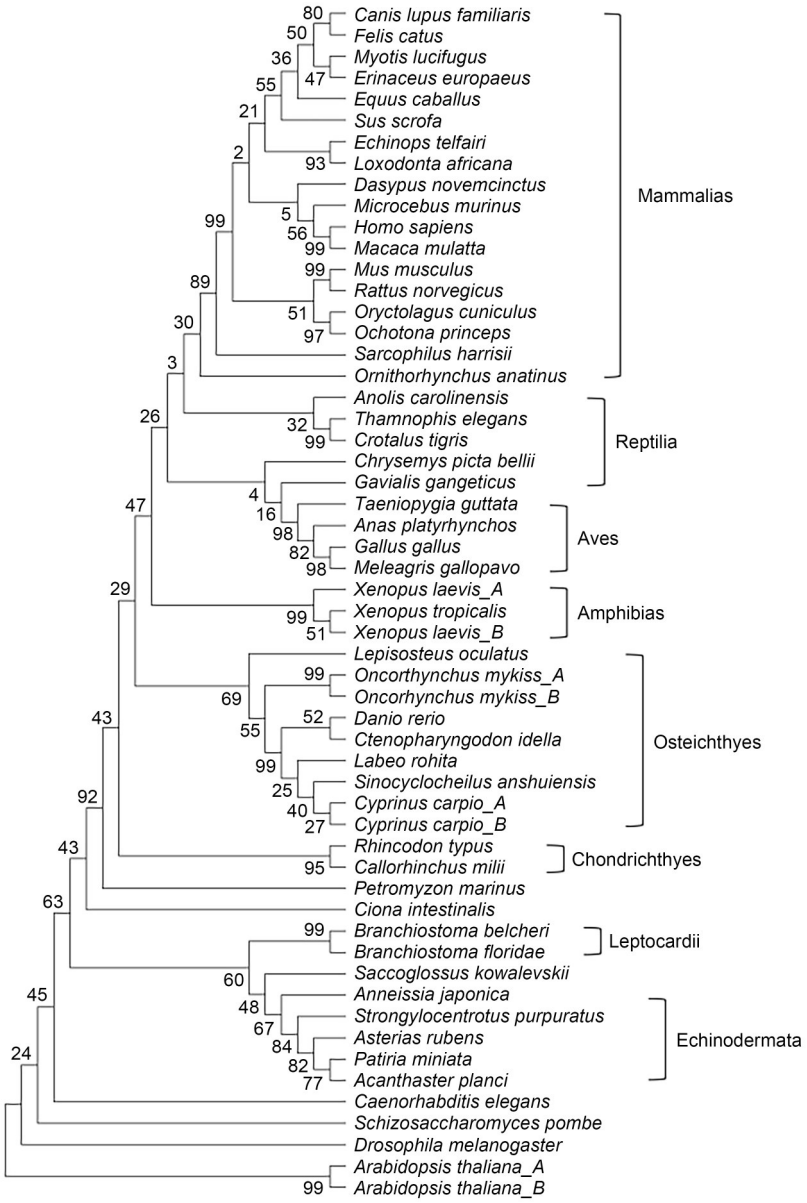

Fig. 2 Phylogenetic tree-derived cladogram of cell division cycle 10 (Cdc10)-dependent transcript 1 (Cdt1) at full length, constructed using 56 Cdt1 protein sequences obtained from 52 organisms (listed in Materials and methods) using MEGA7. Length of line represents evolutionary distance. Number represents bootstrap value, an indication of distance reliability (Bootstrap value of >70 means reliable distance predicted).

components of the pre-replication complex that bring about extreme growth failure (Bicknell et al., 2011). Next, we sacrificed the *cdt1*$^{zju1/zju1}$ mutant fish and dissected some major organs, including the liver, heart, spleen, intestine, testes, and brain, for size/weight comparison (Figs. 5e and 5f). Although these major organs exhibited no obvious phenotypes during the embryonic stages, as shown by normal germ-layer development, their growth, especially that of the testis, was severely arrested in *cdt1*$^{zju1/zju1}$ mutant adult fish (Figs. 5e and 5f).

While impaired replication might be expected to affect growth of all tissues equally, it is noteworthy that in MGS patients, specific tissues are disproportionately reduced in size, particularly the ears and patellae (Nazarenko et al., 2022). The severity observed in the *cdt1*$^{zju1/zju1}$ mutant testis coincides with a high *cdt1* expression level in the reproductive organs revealed in our RNA-seq data (Fig. 4d).

To determine the gene-expression profiles altered by *cdt1* mutation, we extracted total RNA from both

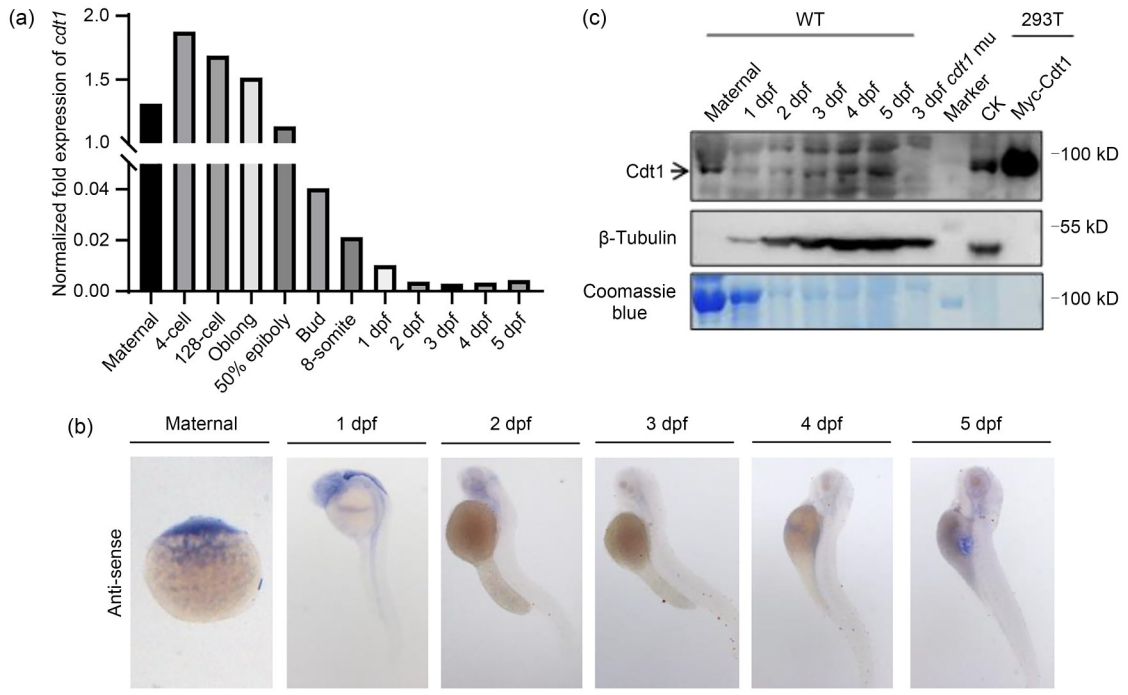

Fig. 3 Expression of *cdt1* in zebrafish. (a) Quantitative real-time polymerase chain reaction (qPCR) analysis of expression levels of the cell division cycle 10 (Cdc10)-dependent transcript 1 (*cdt1*) messenger RNA (mRNA) in maternal, 4-cell stage to 5 dpf zebrafish embryos. (b) Representative whole-mount in situ hybridization (WISH) images showing expression patterns of *cdt1* mRNA in maternal and 1 to 5 dpf zebrafish embryos. (c) Western blot analysis of expression levels of Cdt1 protein in maternal and 1 to 5 dpf zebrafish embryos. Top panel: Cdt1 antibody; middle panel: β-tubulin antibody; bottom panel: Coomassie blue staining. Negative control: *cdt1* mutant (mu); positive control: Myc-Cdt1 overexpression in 293T cells; CK: empty vector; dpf: days post fertilization; WT: wild type.

WT and $cdt1^{zju1/zju1}$ mutant fish at 35 dpf and performed an RNA-seq analysis. Analysis of RNA-seq data revealed that *cdt1* transcript levels were greatly reduced in $cdt1^{zju1/zju1}$ mutant fish (Fig. 4a), suggesting that $cdt1^{zju1}$ mutant messenger RNA (mRNA) is subject to degradation by the nonsense-mRNA-mediated RNA decay pathway (Wittkopp et al., 2009). Surprisingly, detailed sequence analysis revealed that some *cdt1* sequence counts joined exon 1 and exon 3 directly, skipping exon 2 (Fig. 4b). Through a database search, we discovered that two zebrafish *cdt1* transcripts were deposited in the Ensembl database: the first one corresponds to the authentic *cdt1* transcript (designated as *cdt1-201*) which encodes a full length of 678-aa peptide, whereas the second one lacks exon 2 (here in *cdt1-202*) and encodes a 458-aa peptide (Fig. 4b). To confirm the presence of the *cdt1-202* transcript, we designed a pair of genotyping primers that differentiated the two transcripts according to size. *cdt1-201* gave 521 bp and *cdt1-202* yielded a fragment of 371 bp (Fig. 4c, upper panel). These primers were used to amplify corresponding DNA fragments from the complementary DNA (cDNA) generated using total RNA extracted from the WT unfertilized eggs, embryos at 3 dpf, fish at 35 dpf, and adult ovary and testis at 8 mpf. We cloned the PCR products to a T-vector and prepared plasmid DNA from single colonies for sequencing analysis. The sequencing results showed that while *cdt1-201* was the predominant form, transcript *cdt1-202* was present at all stages examined (Fig. 4c, lower panel).

Next, we performed a detailed analysis of the RNA-seq data obtained from heart, intestine, liver, ovary, and testis tissues to specifically identify both *cdt1-201* and *cdt1-202* transcripts. We found that *cdt1-202* was present at a very low level (TPM<0.5) in the liver, ovary, and testis but was undetectable in the heart or intestine (Fig. 4d, upper panel). These results suggest that *cdt1-202* is a genuine transcript. In addition, it appeared that *cdt1-202* exhibited differential expression in different organs (Fig. 4d, upper panel), which might offer one explanation for the different sensitivity of various organs affected in the $cdt1^{zju1/zju1}$ mutant. In relation to this, it is interesting to note that heterogeneous symptoms are observed in patients suffering from

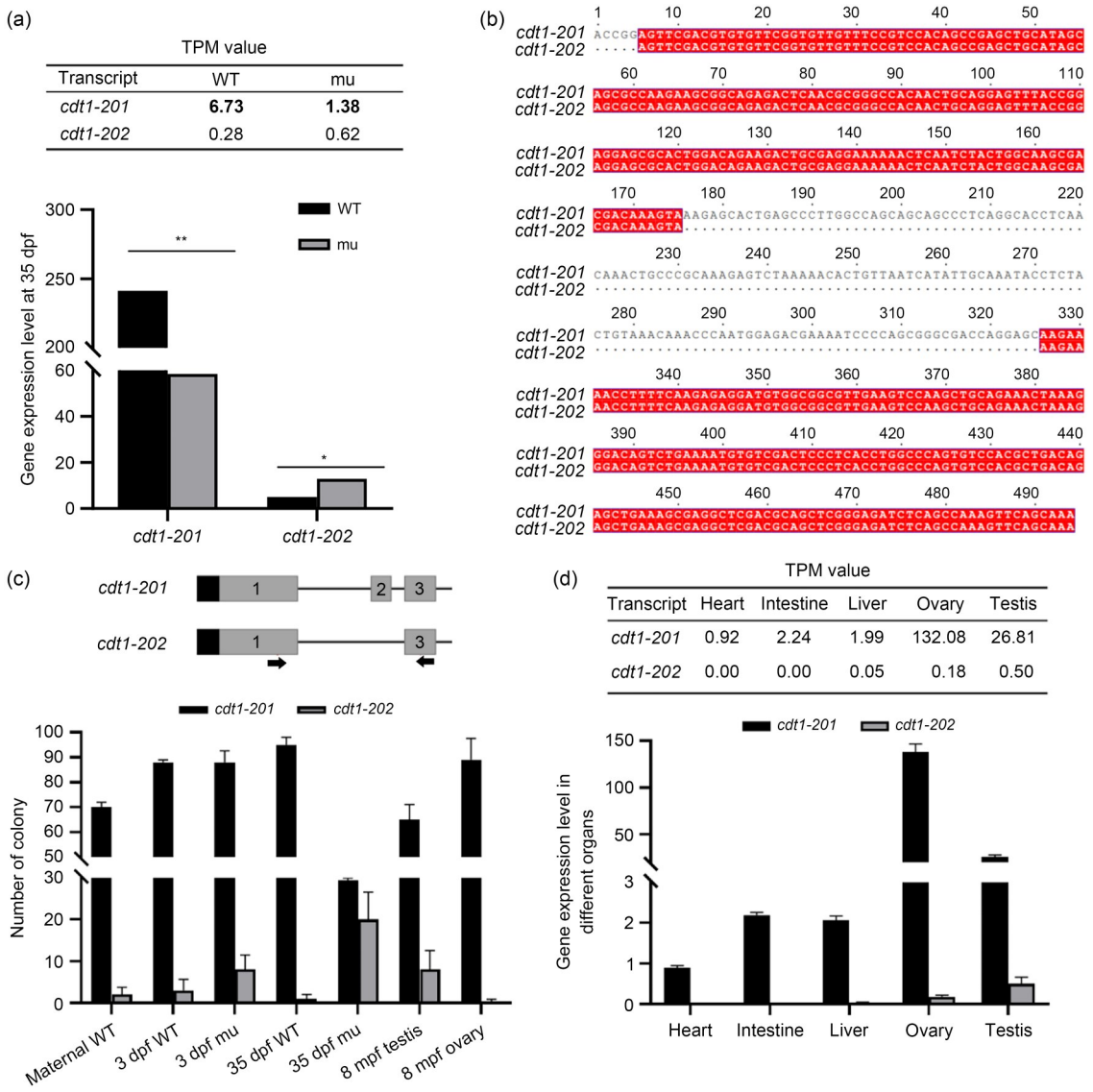

Fig. 4 Transcript cell division cycle 10 (Cdc10)-dependent transcript 1 (*cdt1*)-*202* is barely detectable in wild type (WT) but increases in *cdt1-201* mutant. (a) Expression levels of *cdt1-201* and *cdt1-202* transcripts in WT vs. mutant (mu) fries, obtained by RNA sequencing (RNA-seq). The transcripts per million (TPM) values derived from RNA-seq data are shown (upper panel). (b) Alignment of *cdt1-201* and *cdt1-202* reverse transcription-polymerase chain reaction (RT-PCR) products amplified by primers on exon 1 and exon 3. (c) Expression levels of *cdt1-201* and *cdt1-202* transcripts in WT vs. mu embryos or fries, WT testis, and WT ovary, obtained by RNA reverse transcription followed by single-colony PCR. Positions of primers are indicated. (d) Expression levels of *cdt1-201* and *cdt1-202* transcripts in WT organs, obtained by RNA-seq. The TPM values derived from RNA-seq data are shown in tables (upper panel). Data are expressed as mean± standard deviation (SD), *n*=3 to 10 based on specific tissue size (e.g., three fishes vs. ten hearts). * *P*<0.05, ** *P*<0.01, two-tailed *t*-test. dpf: days post fertilization; mpf: months post fertilization.

MGS. It is also noteworthy that *cdt1-202* seems to be enriched in WT testis tissue, in comparison to WT ovary tissue (Fig. 4d, upper panel), a possible biological significance worth pursuing in the future.

Considering that the exon 2 mutation in *cdt1*$^{zju1/zju1}$ exists only in the coding frame of *cdt1-201* and not that of *cdt1-202* (Fig. 5a), we wondered if there were any alterations to *cdt1-202* transcript levels. We first compared the transcript levels of *cdt1-202* in the RNA-seq data obtained from WT and *cdt1*$^{zju1/zju1}$ fish at 35 dpf, and found an approximately 2.2-fold increase in the *cdt1*$^{zju1/zju1}$ mutant (Fig. 4a). We next compared the levels of *cdt1-202* between WT and *cdt1*$^{zju1/zju1}$ at 3 and 35 dpf through single-colony DNA sequencing (Fig. 4c).

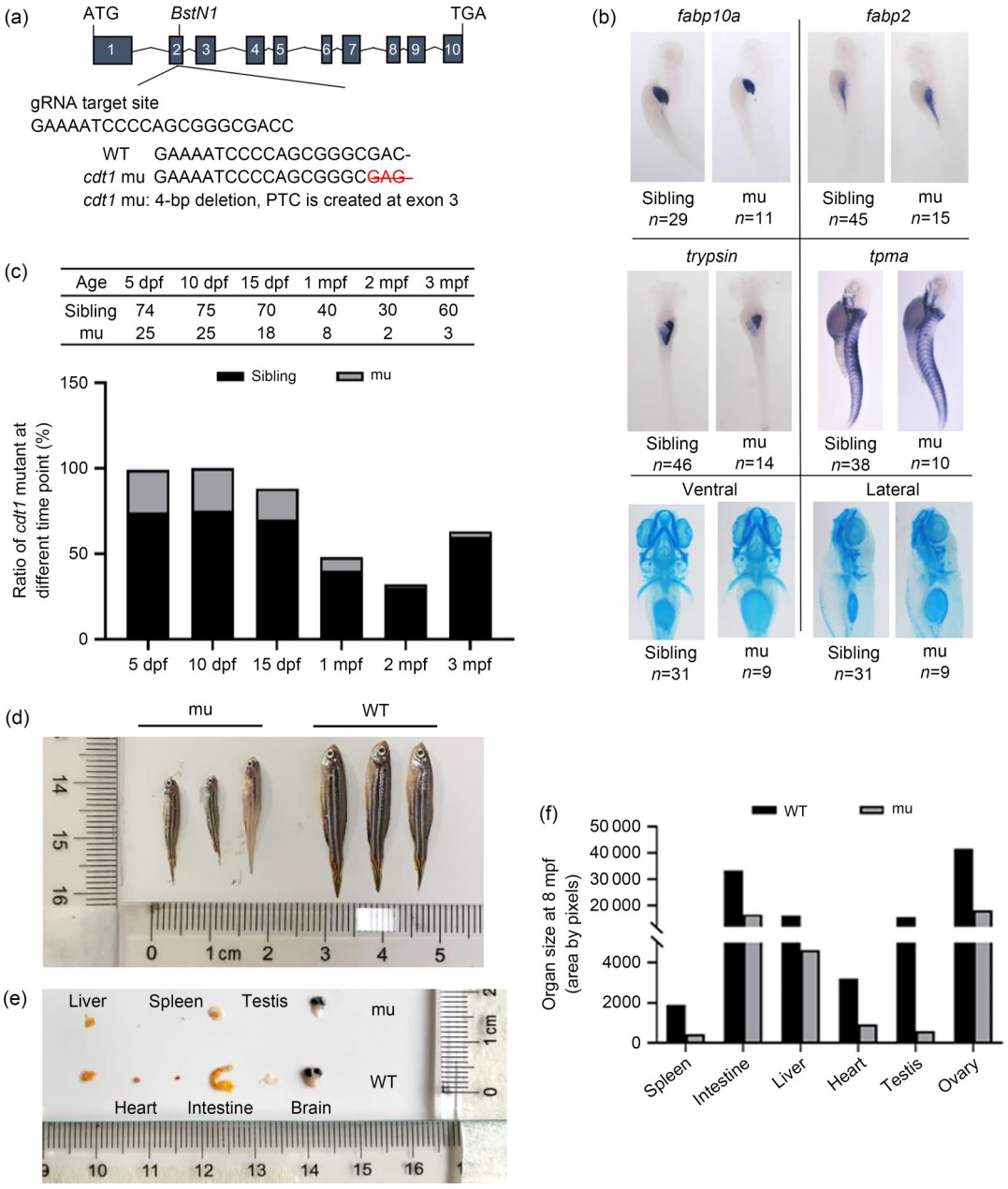

Fig. 5 Zebrafish cell division cycle 10 (Cdc10)-dependent transcript 1 (*cdt1*)-*201* mutant is viable but suffers from growth retardation and infertile. (a) Schematic diagram depicting generation of the *cdt1-201* mutant (mu) with guide RNA (gRNA) targeting of exon 2 of *cdt1-201*. (b) Expression of molecular markers from the three germ layers in 5 dpf wild type (WT) siblings (left panel) and mutant (mu) (right panel). Fraction represents number of observed phenotypes/total embryos scored. (c) Table shows the actual numbers of viable siblings and mutants (mu) scored from 5 dpf to 3 mpf. Bar chart shows the survival scores (%) of mutant from 5 dpf to 3 mpf. (d, e) Comparison of 8 mpf WT and mutant (males) in overall body size and size of various major organs including liver, heart, spleen, intestine, testis, and brain. Scale is in cm. Sample number in each group is three. (f) The mean area of each organ is tabulated into a bar graph. Standard deviation is not calculated due to too small sample size (*n*=3, for each organ). dpf: days post fertilization; mpf: months post fertilization.

We found that the frequency of *cdt1-202* colonies, compared to that in the WT control, increased obviously in *cdt1*$^{zju1/zju1}$ at 3 dpf (4 folds) and dramatically at 35 dpf (20 folds) (Fig. 4c), likely due to the steep reduction of *cdt1-201* transcript levels in *cdt1*$^{zju1/zju1}$ that altered the template ratio of *cdt1-201* versus *cdt1-202* (Fig. 4a). Thus, we would expect to detect Cdt1-202 on western blot if 35-dpf mutant protein samples were

used instead of 3-dpf samples (Fig. 3c). This observation highlights a possible link between mutant survival through functional compensation by Cdt1-202, a hypothesis worth probing in the future. However, dosage of the upregulated *cdt1-202* might not be sufficient to compensate for normal growth processes. Considering the infertility phenotype suffered by the *cdt1*$^{zju1/zju1}$ mutant, we are currently unable to exclude the possibility that Cdt1-202 acts as a dominant-negative regulator of testis development.

In summary, using zebrafish as a model system, our work puts forward three preliminary but interesting discoveries on *cdt1*, a well-studied player in the pre-replication complex. First, it is worth pursuing whether the Cypriniformes-specific N-terminal has a function or act as an "evolutionary tag." Second, it is of great interest whether *cdt1-202* transcript exists in other organisms and what its biological functions might be. Third, because the zebrafish *cdt1* mutant exhibits phenotypes that mimic MGS patients, this genetic mutant may serve as a model for drug screening to treat or alleviate MGS.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

The sequencing data were deposited in the NCBI Sequence Read Archive (submission ID: SUB13301727; BioProject ID: PRJNA970210).

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Author contributions

Yinan HE, Yong WANG, Yanqing ZHU, and Li Jan LO performed the conceptualization and methodology; Yinan HE performed the investigation; Yong WANG, Yinan HE, and Li Jan LO contributed to formal analysis; Li Jan LO contributed to the resources; Yinan HE was responsible for the data curation; Yinan HE and Li Jan LO wrote the original draft; Yong WANG and Li Jan LO contributed to visualization and funding acquisition. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yinan HE, Yong WANG, Yanqing ZHU, and Li Jan LO declare that they have no conflict of interest.

All animal experiments were approved by the Animal Care Committee of Zhejiang University, and all methods were performed in accordance with the Guidelines for the Care and Use of Animals for Research and Teaching at Zhejiang University (ETHICS CODE Permit No. ZJU20220031).

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Supplementary information

Materials and methods