1 Method and materials

1.1 Phylogenetic tree construction and analysis

To obtain the sequences for phylogenetic analysis, zebrafish Cdt1 (XP_695164.3) was used as query to search against the non-redundant protein database using the phi-blast algorithm (NCBI), with iterated searches until no further significant hits were found. Hits with E-value > 0.005 were first removed, followed by redundant sequences, spliced variants, and hits with too short lengths. From that, sequences from 52 representative species were selected and further screened for the presence of Cdt1-geminin interaction domain and Cdt1-MCM binding domain. At the same time, the presence of homologous genes encoding Cdt1 in the selected species was also checked. Eventually, 56 Cdt1 sequences obtained from 52 organisms (Table 1) were selected for subsequent analysis, where two copies were found in *Xenopuslaevis*, *Oncorhynchus mykiss*, *Cyprinus carpio*, and *Arabidopsis thaliana*, respectively. Multiple sequence alignment was calculated by ALIGN BY MUSCLE[1]. TrimAL[2] was used to remove gaps before tree construction. Maximum likelihood trees were then constructed using the MEGA7 toolbox[1] with a bootstrap testing for 1,000 times.

1.2 Mutant line generation and fish maintenance

Wildtype zebrafish strain AB was used in this study, and mutant was generated using this genetic background.Adult fish of both sex were used unless otherwise stated. Mutant $cdt1^{zju1}$disrupting the *cdt1-201* transcript was generated using CRISPR-Cas9 strategy with a gRNA targeting exon2 of the zebrafish *cdt1* gene following protocol described[3]. Mutant was identified via PCR with ID primers *cdt1*-ID-F and *cdt1*-ID-R, followed by restriction digestion by *BstN1*. The term 'siblings' used as control in some experiment refers to the $cdt1^{+/+}$and $cdt1^{zju1/+}$progenies laid by the same parent pair, while mutant means $cdt1^{zju1/zju1}$ homozygous mutant obtained within the laid population.

1.3 Whole-mount in situ hybridization (WISH)

WISH probes were labeled with DIG RNA Labeling Mix (Roche Diagnostics). For anti-sense and sense *cdt*1 probes, primers were shown in Table S1. Probes *fabp10a, fabp2,trypsin*, *tmpa*[4], and the method of Alcian blue staining were used as previously described[5] and WISH protocols were executed as described[6].

1.4 Real-time quantitative PCR (qPCR)

DNase I (NEB) treated total RNA was subjected to reverse transcription (INVITROGEN) by oligo-dT and random hexamer. SYBR qPCR (Vazyme) was performed on a CFX96 Real-Time System (Bio-Rad) according to manufacturer's instructions. All reactions were run in triplicates, using 18S rRNA as internal reference for normalization. The primers for *cdt1* and *18S* rRNA are listed in Table S1. Statistical analysis Student's t-test was used for statistical comparisons (*P<0.05; **P<0.01; n.s.: no significant difference). The Bio-Rad software was used to determine the correlation between *cdt1* and *18S* rRNA.

1.5 Western blot analysis

Embryos of desired stages were first deyolked using NO.21 needle and spun at 12,000g for 1 min. Precipitates were lysed in SDS lysis, supplemented with 1× cOmplete Protease Inhibitor Cocktail (Roche Diagnostics). The zebrafish FL *cdt1* plasmid (mentioned above) was transfected into 293T cells for 48 h. Cells in six-well plate were washed twice with PBS, and 200uL SDS lysis buffer was added to collect the sample. Protein samples were used immediately for western blot analysis or stored at −20 ℃ for later use. Primary antibodies: zebrafish Cdt1 guinea pig antibody was generated by ABclonal

(Wuhan, China) (1:400) and β-Tubulin from Huaan (Hangzhou, China) (1:5,000); secondary antibodies used were from ABclonal (anti-guinea pig) and Huaan (anti-mouse), both at 1:10,000 respectively.

1.6 Survival rate scoring

Heterozygous adult pairs of *cdt1*$^{zju1/+}$ were allowed to self-cross. Dead embryos were removed daily and 100 progenies were sacrificed randomly at each time point and genotyped with ID primers mentioned above. From 15 days post fertilization (dpf) onwards, less than 100 samples were assayed due to limited progeny numbers (Fig. 4c).

1.7 Single colony PCR

Total RNA was extracted from the desired stages and DNase I treated. Reverse transcription (INVITROGEN) was carried out with oligo-dT mixed random hexamer, followed by amplification with *cdt1*isotype primers (Table S1). After purification (SANGON, CHINA), PCR products were cloned into T-vector. Upon transformation and plating, colony PCR was carried out with the same primer pair. The frequency of transcript *cdt1-201* or *cdt1-202* was scored by PCR product sizes, where 553 bp for *cdt1-201* and 317 bp for *cdt1-202*. A total 96 colonies were picked for each stage and insert-positive colonies were tabulated.

1.8 RNA-seq

After DNase 1 (NEB) treatment, LiCl and absolute alcohol were added to total RNA and allowed overnight precipitation.Purified RNA samples were subjected to gel analysis and OD measurement where 260/280 values fall between 1.8-2.1 Verified samples were sent to and processed by ANNOROAD Gene Technology (Beijing, China) for RNA-seq. Upon obtaining the transcriptome data using the transcript database on Ensemble as reference, the sequence, ReadsNum and TPM values of *cdt1-201* and *cdt1-202* were derived respectively[7]. The sequencing data were deposited in the NCBI Sequence Read Archive (submission ID: SUB13301727; BioProject ID: PRJNA970210).

Table S1 Primers used for colony PCR, genotyping, qPCR, gRNA and WISH probe generation

Primer name	Sequence (5' to 3')
GSP-1	CAAGGTTAAGCAAGGGGTCC
GSP-2	TTCTCTGAATCCTCCTATT
cdt1-ID-F	GACCCATTAGGATGTTTCAA
cdt1-ID-R	AAAGTTCAGCAAAGAAAGGC
cdt1-qPCR-F	AAGTTAGTCTAGGGAAGACC
cdt1-qPCR-R	GCGGTGATCAATAAACCGAG
*18S*rRNA-F	CCAACCTGGTTGATCCTGCCAGTA
*18S*rRNA-R	CCTTGTTACGACTTCACCTTCCTCT
cdt1-isotype-F	ATGTGAACATTGAGGGCTTA
cdt1-isotype-R	GCCTTTCTTTGCTGAACTTT
WISH-sense-F	TTTGTTTACAGTAGAGGTAT
WISH-sense-R	TAATACGACTCACTATAGGGAGCTCGTATTACTGATTATT
WISH-antisense-F	TAATACGACTCACTATAGGGTTTGTTTACAGTAGAGGTAT
WISH-antisense-R	AGCTCGTATTACTGATTATT
cdt1 gRNA	GAAAATCCCCAGCGGGCGACC

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