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Generation of *cdk5* mutant zebrafish reveals its essential role in early development and anterior commissure formation

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Abstract

The correct development of the central nervous system (CNS) is essential for normal functioning. Numerous genes contribute to this process, including cyclin-dependent kinase 5 (Cdk5). We previously investigated the role of *cdk5* in zebrafish neurogenesis using an antisense morpholino oligonucleotide-mediated knockdown (KD) approach. In the present study, we generated *cdk5* mutant zebrafish using CRISPR/Cas9 genome-editing technology. Since different phenotypes have been reported in KD and knockout zebrafish for some genes, we tested these comparisons in the case of *cdk5*. The ectopic Rohon–Beard neurons and cap motor neurons reported in KD embryos were not observed in *cdk5*$^{-/-}$ zebrafish. Furthermore, abnormalities in neuromuscular junctions and facial motor neuron migration, previously reported in *Cdk5* knockout mice, were absent in the mutants. However, significant abnormalities in anterior commissure formation were observed in *cdk5* mutant zebrafish. Therefore, *cdk5* may be required for AC formation in zebrafish, and *cdk5* mutant zebrafish may be a valuable model for studying the role of *cdk5* in CNS development.

Introduction

The zebrafish (*Danio rerio*) possesses several biological advantages as a model organism and has been widely used across diverse research fields (Howe et al., 2013; Teame et al., 2019). Zebrafish embryos develop ex utero and are highly transparent, allowing direct observation of tissue and organ formation, including the nervous system, from the single-cell stage. Moreover, established genome-editing techniques such as CRISPR/Cas9 enable the efficient generation of mutants targeting specific genes. Together, these characteristics make zebrafish a powerful system for in vivo analysis of gene function during development.

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that belongs to the cyclin-dependent kinase family. However, unlike other Cdks, it is not directly involved in cell cycle regulation (Requejo-Aguilar, 2023). Cdk5 is ubiquitously expressed, predominantly in neurons, where it plays a crucial role in the regulation of neurogenesis and neuronal function in post-mitotic cells. Cdk5 is catalytically inactive on its own but is activated by binding to the neuron-specific regulatory subunits p35 or p39, which are structurally related to cyclins. Activated Cdk5 phosphorylates several axon-formation-related factors, including collapsin response mediator proteins (CRMPs), thereby regulating cytoskeletal dynamics and axon guidance responses (Brown et al., 2004). Cdk5 acts downstream of the semaphorin 3 (Sema3) signaling pathway. Specifically, in the Sema3A cascade, Cdk5 phosphorylates CRMP2 at Ser522, leading to axonal retraction (Uchida et al., 2005).

We previously reported *cdk5* knockdown (KD) phenotypes using an antisense morpholino oligonucleotide (AMO)-mediated KD approach (Morimura et al., 2013), including the abnormal positioning of Rohon–Beard (RB) neurons and primary motor neurons in the developing spinal cord (Morimura et al., 2013; Tanaka et al., 2012). However, as AMOs can induce off-target effects through p53-dependent cell death (Robu et al., 2007; Rossi et al., 2015), we generated *cdk5* mutant zebrafish lines. Furthermore, we recently reported abnormal anterior commissure (AC) formation in *crmp2* mutant zebrafish, a substrate of Cdk5. The defective AC formation observed in *crmp2* knockout (KO) zebrafish was rescued by wild-type (WT) *crmp2* mRNA, but not by non-phosphorylatable *CRMP2* mRNA (Guo et al., 2022), suggesting that Cdk5-mediated phosphorylation of CRMP2 is required for AC formation. Furthermore, lissencephaly-7 with cerebellar hypoplasia (LIS7) lacks corpus callosum because of a homozygous mutation in the *Cdk5* gene (Magen et al., 2015) and Emx1–Cre-mediated Cdk5 conditional KO mice (Hirota et al., 2007). *Cdk5*−/− mice also exhibit defective formation of neuromuscular junctions (NMJs) (Fu et al., 2005) and migration defects in facial motor neurons (Ohshima et al., 2002). Therefore, in the present study, we investigated the role of *cdk5* in spinal cord development and AC formation, as well as in NMJ formation and positioning of facial motor neurons.

Materials and Methods

Animals

Zebrafish were maintained according to standard procedures (Westerfield, 2007). The RIKEN Wako (RW) WT strain was obtained from the Zebrafish National BioResource Center of Japan (http://www.shigen.nig.ac.jp/zebra/). The *Tg(islet1:GFP)* line was generated in an RW background and provided by the Zebrafish National BioResource Center of Japan.

Genome editing using the CRISPR/Cas9 system

CRISPRdirect (https://crispr.dbcls.jp/) was used to design the crRNA sequence zebrafish *cdk5* gene. Synthetic CRISPR RNA (crRNA; 5′-CATCCAGTCGGACTCGTTTGAGG-3′), Cas9 protein, and transactivating CRISPR RNA (tracrRNA) were purchased from FASMAC. Both crRNA and tracrRNA were dissolved in sterilized water at 1000 pg/nL and stored at −20 °C. Cas9 protein was dissolved in a specified dilution buffer (10 mM Tris–HCl (PH7.5), 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT) at 5000 pg/nL and stored at −80 °C. A mixture containing 25 pg each of crRNA and tracrRNA with 250 pg of Cas9 protein was co-injected into one- to two-cell-stage fertilized eggs derived from WT zebrafish.

Genotyping

For polymerase chain reaction (PCR) amplification of the *cdk5* locus from adult zebrafish, fin clips were incubated in lysis buffer containing proteinase K (Wako) for 24 h at 55 °C. When both genotyping and phenotypic analyses were performed using embryos, each embryo was bisected; half was used for genotyping, and the other half was used for analysis (Supplementary Fig. 1A). Genomic DNA was extracted by ethanol precipitation and used as the PCR template. The PCR products were cloned into the T-vector pMD19 (Simple) (Takara), and the cloned samples were sequenced using the M13 primer M4 (Irion et al., 2014). After the introduction of mutations at the target site using the sequence of F0 fish, the F1 generation was obtained by crossing the *cdk5* mutant F0 zebrafish and WT fish. We then genotyped the F1 fish to identify the mutant F1 zebrafish. For genotyping of the *cdk5* mutant Line 1, specific primers for both the WT and mutant alleles were used (Fig. 1A and Supplementary Fig. 1B).

Immunohistochemistry

Immunohistochemistry was performed on whole-mount embryos at 28, 50, and 72 h post fertilization (hpf). In the staining experiments, embryos were raised in fish water (de-chlorinated freshwater) containing 0.003% N-phenylthiourea (PTU; Nacalai Tesque, Kyoto, Japan) at 12 hpf (Westerfield, 2007). Briefly, embryos were fixed overnight in 4% paraformaldehyde at 4 °C. Each sample was washed thrice in 0.1% PBT (PBS containing 0.1% Triton X-100) and blocked in 0.1% PBT supplemented with 2% blocking reagent (Roche) for 1 h at room temperature before incubation with the primary antibody. For primary antibodies, mouse anti-acetylated α-tubulin (1:2000; Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-SV2 (1:100, DSHB) were used. For secondary antibodies, Histofine Simple Stain MAX-PO (M) (Nichirei) and Alexa Fluor 488- and 647-conjugated subclass-specific antibodies (1:500; Invitrogen) were used. For

tubulin staining, the signal was developed using DAB tablets for 1 h at room temperature, followed by a 5-minute reaction with 1:1000 hydrogen peroxide to visualize the staining (Santos et al., 2018). To visualize postsynaptic sites at NMJs, Alexa Fluor 488-conjugated α-bungarotoxin (α-BTX; 1:50; Thermo Fisher Scientific) was used, as previously described by Nozawa et al. (2017).

Imaging

For conventional light and fluorescence microscopy, a BX50 microscope (Olympus) equipped with UPlanApo 20× (NA 0.70) and UPlanApo 40× (NA 0.75) objectives were used. To analyze the *Tg(isl1:GFP)* strain, an FV1000 confocal laser scanning microscope (Olympus) equipped with UPlanSApo 20× (NA 0.75) and UPlanSApo 40× (NA 0.90) objectives and a Zeiss LSM510 laser scanning confocal microscope equipped with an Achroplan 20× (NA 0.50) water-immersion objective lens were used. Images were obtained from the ventral views using z-stack analysis. Fiji (ImageJ, Madison, WI, USA) was used to measure staining areas (SV2, α-BTX), and all measurements were obtained from the same somite. Images were processed using Adobe Photoshop and Adobe Illustrator (Adobe Systems, San Jose, CA, USA).

Statistical analysis

Data are expressed as means ± standard errors of the mean. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using Student's *t*-test in Microsoft Excel. For the analysis of AC, statistical significance among the three genotypes (*cdk5*+/+, *cdk5*+/−, and *cdk5*−/−) was evaluated using the chi-square (χ^2) test. Subsequent pairwise comparisons between specific genotypes (*cdk5*+/+ vs. *cdk5*+/− and *cdk5*+/+ vs. *cdk5*−/−) were conducted using Fisher's exact test. All statistical tests were performed using Microsoft Excel, with $p < 0.05$ considered significant.

Results

Generation of *cdk5* mutant zebrafish by genome editing using the CRISPR/Cas9 system

We designed a crRNA to introduce a double-strand break into exon 2 of the zebrafish *cdk5* gene, which was expected to cause a frameshift near the 5′ end of the mRNA, resulting in premature translation termination. Microinjection of the Cas9 protein and guide RNA into one- to two-cell-stage embryos yielded F0 individuals, of which 73% (n = 11) carried mutations at the *cdk5* target site. To identify heritable mutations, F0 *cdk5* mutant fish were crossed with WT fish to obtain the F1 progeny carrying heterozygous alleles. Sequence analysis revealed two independent *cdk5* mutant lines with frameshift mutations leading to premature stop codons. One line carried a six-nucleotide insertion (+6) that introduced a stop codon (line 1), whereas the other had a two-nucleotide insertion (+2) and a one-nucleotide substitution, resulting in a frameshift (line 2). These mutations were predicted to generate truncated proteins consisting of 36 amino acids (AA) and in Line 1 and 54 AA in Line 2, respectively, compared with 292 AA in the WT allele (Supplementary Fig. 2). Line 1 was primarily used for the subsequent analyses. We examined *cdk5* mRNA expression levels in *cdk5*+/+ and *cdk5*-/- larvae at 72 hpf. *cdk5* mRNA expression was significantly lower in *cdk5*-/- mutants compared with wild-type controls, indicating that the mutant mRNA undergoes nonsense-mediated decay (data not shown). To obtain F2 offspring carrying biallelic *cdk5* knockout alleles, F1 heterozygous fish from each line were intercrossed. Homozygous *cdk5*−/− zebrafish from line 1 exhibited embryonic lethality 4 days post-fertilization (dpf) (Fig. 1B). Examination of external morphology and motor behavior in line 1 revealed no morphological differences or differences in motor behavior among the genotypes (morphology: Fig. 1C). In the F2 generation of line 1, all genotypes were observed until 3 dpf (+/+: 8%; +/−: 69%; −/−: 23%), confirming the survival of *cdk5*−/− larvae up to this stage. However, *cdk5*−/− mutants were not detected beyond 4 dpf (Fig. 1B). Thus, *cdk5*−/− zebrafish exhibited early lethality, beginning at 4 dpf. Therefore, *cdk5* mutant zebrafish from line 1 were used for subsequent analyses.

cdk5 KO zebrafish did not exhibit ectopic neuronal localization in the spinal cord

A previous study using *cdk5* morphant zebrafish demonstrated that RB neurons (Tanaka et al., 2012) and CaP motor neurons (Morimura et al., 2013) migrate to ectopic positions in the spinal cord, indicating that *cdk5* regulates the migration of these neurons. To determine whether *cdk5* mutant zebrafish exhibit neuronal migration defects in the spinal cord, we examined neuronal localization using tubulin staining. In contrast to the morphant phenotypes, *cdk5*−/− zebrafish showed no abnormal localization of RB or CaP motor neurons in the spinal cord (Supplementary Fig. 3).

Pre- and postsynaptic densities were not significantly altered in *cdk5* mutant zebrafish

Dhavan and Tsai (2001) have suggested the involvement of Cdk5 in synapse formation. However, the role of Cdk5 in synapse formation in zebrafish remains unclear. To investigate the function of *cdk5* during synaptogenesis, we analyzed the pre- and postsynaptic structures of NMJs at 72 hpf in line 1 using SV2

antibody and fluorophore-conjugated α-BTX as pre- and postsynaptic markers, respectively. Presynaptic regions largely matched with postsynaptic regions in both *cdk5*+/+ and *cdk5*−/− zebrafish. Although the densities of the presynaptic regions in either genotype did not differ, the postsynaptic regions in *cdk5−/−* mutants appeared denser than those in *cdk5*+/+ zebrafish; however, no statistically significant differences were detected between the two genotypes (Supplementary Fig. 4A).

cdk5 KO zebrafish did not exhibit ectopic localization of facial motor neurons

A previous study on *Cdk5* KO mice has reported that facial nerves in the hindbrain are ectopically located, indicating that Cdk5 regulates neuronal migration in the developing mouse hindbrain (Ohshima et al., 2002). To determine whether this function of cdk5 is conserved in zebrafish, we examined the cranial neuron localization in *cdk5−/−* zebrafish using the *isl1:GFP* transgenic line. Facial motor neurons were analyzed at 60 hpf. In all examined individuals, facial motor neurons were correctly positioned in the rhombomere 6–7 (r6–r7) region at 60 hpf, consistent with the WT localization. Moreover, other cranial neurons, including vagus and trigeminal neurons, also migrated to normal positions (Supplementary Fig. 4B). Thus, *cdk5* loss did not cause ectopic migration of facial motor neurons in zebrafish.

cdk5 mutant disrupts anterior commissure formation

To analyze the *in vivo* role of *cdk5* in zebrafish embryonic forebrain development, we examined the AC by immunostaining the heads of 30 hpf *cdk5* mutant embryos with an anti-acetylated α-tubulin antibody. Although the overall brain morphology appeared normal, abnormalities in anterior commissure (AC) structure were observed in a subset of embryos. Specifically, axon bundles forming the AC either failed to reach the midline or displayed substantial defects (Fig. 2A). The rate of AC malformation was 4% (n = 25) in *cdk5*+/+, 27% (n = 30) in *cdk5*+/−, and 33% (n = 16) in *cdk5*−/− embryos, indicating significant differences among the three *cdk5* genotypes ($\chi^2(2) = 7.527$, $p = 0.0232 < 0.05$; chi-square test). Furthermore, pairwise comparisons using Fisher's exact test revealed significant differences between *cdk5*+/+ and *cdk5*+/− ($p = 0.0307 < 0.05$) as well as between *cdk5*+/+ and *cdk5*−/− ($p = 0.0094 < 0.01$) (Fig. 2B).

Discussion

In the present study, we generated *cdk5* mutant zebrafish lines using the CRISPR/Cas9 genome editing method. *cdk5−/−* fish exhibited early lethality beginning at 4 dpf (Fig. 1B), which is similar to the phenotype reported in *Cdk5*−/− mice (Ohshima et al., 1996). We then analyzed the positioning of RB and CaP motor neurons in the developing spinal cord and the abnormalities previously observed in c*dk5* KD zebrafish. Unlike the phenotypes of antisense AMO-induced *cdk5* KD, RB, and CaP motor neurons in *cdk5* KO larvae did not exhibit abnormal positioning (Supplementary Fig. 3). Phenotypic differences between AMO-mediated KD and genetic KO can be explained by at least four factors. First, the AMO-mediated KD strategy may have off-target effects ((Robu et al., 2007; Rossi et al., 2015). Second, other *cdks* may compensate for the loss of *cdk5* because of the high AA sequence similarity between *cdk5* and other *cdks*, particularly *cdk2*. Third, *cdk5* may function as a maternal effect gene. Since *cdk5−/−* zebrafish exhibited lethality after 4 dpf (Fig. 1B), we obtained *cdk5−/−* embryos by crossing *cdk5+/−* adults and analyzing them at 3 dpf. Therefore, maternally derived *cdk5* mRNA may act during early development, thereby reducing the likelihood of phenotypic abnormalities at this stage. *cdk5* mRNA expression in zebrafish embryos has been reported across early development, from the zygote stage to 5 dpf (White et al., 2017). Other *cdks* that are active in early embryonic stages may show maternal effects. Fourth, abnormal phenotypes are absent in knockout models owing to genetic compensation (El-Brolosy et al., 2019). This possibility can be addressed through transcriptomic approaches such as RNA sequencing by comparing *cdk5+/+* and *cdk5−/−* zebrafish.

We also examined phenotypes previously reported in *Cdk5*$^{-/-}$ mice, including defective NMJ formation (F et al., 2005). A previous in vivo examination of the NMJ phenotype in *Cdk5*−/− mice demonstrated that the bandwidth of the acetylcholine receptor (AChR) endplate is enlarged in *Cdk5*$^{-/-}$ diaphragm (Fu et al., 2005). Furthermore, AChR clusters were significantly larger in cultured myotubes lacking Cdk5 after agrin treatment, suggesting that Cdk5 may negatively regulate AChR cluster size (Fu et al., 2005; Lin et al., 2005). Although the difference was not statistically significant, we observed a tendency toward increased densities in the postsynaptic regions (Supplementary Fig. 4A). This will be partly matched with phenotype of *Cdk5*−/− mice. We also examined the positioning of facial motor neuron which was defective in *Cdk5*−/− mice (Ohshima et al., 2002). However, facial motor neuron positioning was not affected in *cdk5−/−* zebrafish (Supplementary Fig. 4B). Phenotypic discrepancies between *cdk5-/-* zebrafish and *Cdk5-/-* mice may be explained by differing degrees of maternal contribution. However, testing this maternal effect is experimentally challenging due to the early lethality of *cdk5-/-* zebrafish.

In the present study, both *cdk5*+/− and *cdk5*−/− zebrafish exhibited a higher frequency of AC abnormalities than *cdk5*+/+ fish. KD or genetic knockout of *crmp2* results in abnormal AC formation. Furthermore, this phenotype can be rescued by the introduction of WT *crmp2* mRNA, whereas S522A *crmp2* mRNA, which cannot be phosphorylated at Ser522, the Cdk5 target site, failed to rescue this

phenotype, suggesting that *cdk5*-mediated phosphorylation of crmp2 is required for axon outgrowth (Guo et al., 2022). Collectively, our findings support the involvement of *cdk5* in AC formation during zebrafish development. Because CRMP2 is a specific substrate of *Cdk5* (Uchida et al., 2005) and crmp2 phosphorylation by *cdk5* is essential for AC formation (Gao et al., 2022), *cdk5−/−* zebrafish may also show defects in AC formation. Thus, Cdk5 may have a common role in axonal formation, which connects the right and left brain, the corpus callosum in mammals (mice and humans), and the AC in vertebrates (zebrafish).

In the present study, we investigated various aspects of nervous system development in *cdk5−/−* zebrafish. The transparency and external embryonic development of zebrafish allowed detailed observation from the one-cell stage, enabling the examination of genes essential for early viability such as *cdk5*. Therefore, this system provides a valuable platform for elucidating molecular mechanisms underlying cdk5-mediated neural development in future studies.

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Ethical Approval

The zebrafish used in accordance with protocols approved by the Institutional Animal Care and Use Committee at Waseda University.

Conflict of Interest Statement

The authors have no conflict of interests.

Data availability statement

The data that support the findings of this study will be available on request from the corresponding author upon reasonable request.

Funding statement

There is no funding source in this research.

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Figure Legends

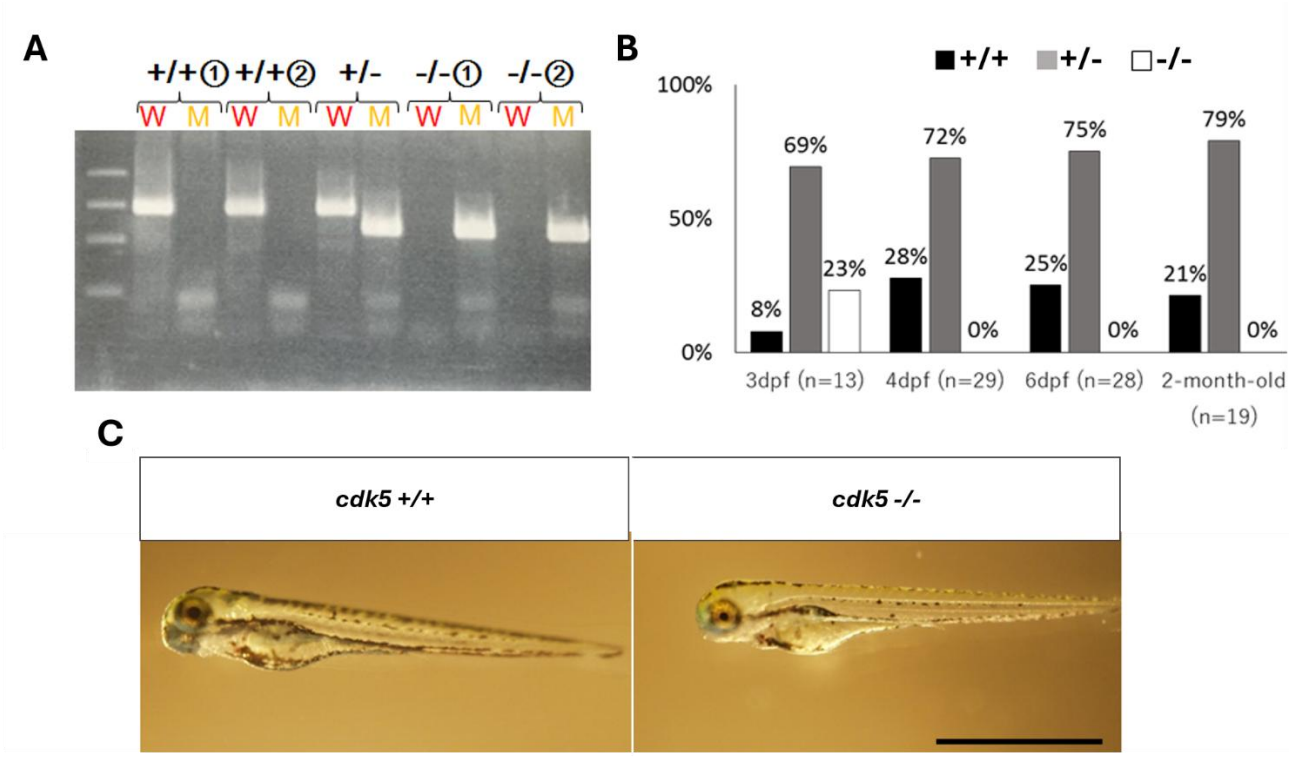


Figure 1. Genotype identification and early lethality in *cdk5* mutant zebrafish. (A) Electrophoresis results following polymerase chain reaction amplification using specific *cdk5* primer sets (W: WT *cdk5* detection primer set [white arrow]; M: mutant *cdk5* detection primer set [red arrow]). Five samples (1–5) from 72 hpf embryos obtained from *cdk5*+/− × *cdk5*+/− mating were genotyped. Genotypes of each sample are shown below the gel image. (B) *cdk5−/−* zebrafish exhibited early larval lethality beginning at 4 dpf. The graph shows the percentage of genotypes among embryos derived from intercrosses of *cdk5+/−* fish (black bar: +/+; gray bar: +/−; white bar: −/−). Sample sizes: n = 13 (3 dpf), n = 29 (4 dpf), n = 28 (6 dpf), and n = 19 (2 months old). (C) Lateral views of embryos from each genotype at 72 hpf. Scale bar, 1.0 mm.

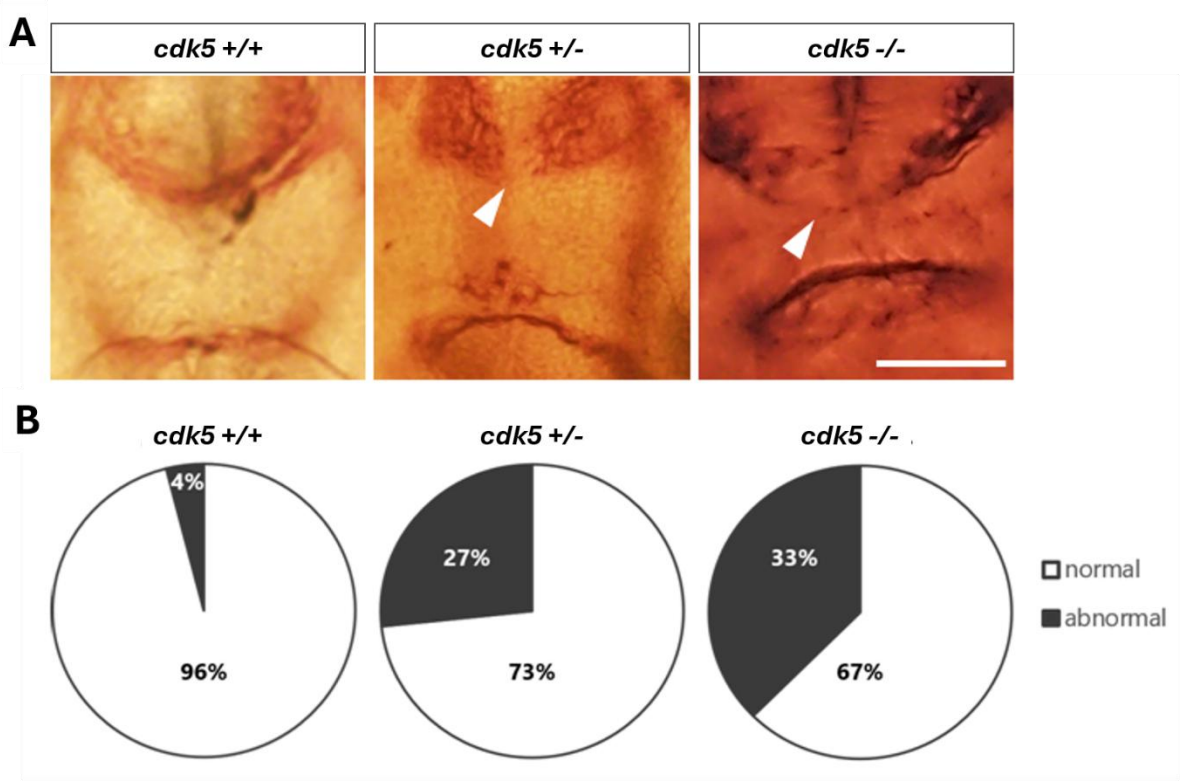


Figure 2. *cdk5* knockout disrupts anterior commissure formation in zebrafish embryos. (A) Representative ventral images of zebrafish embryonic heads at 30 hpf immunostained with anti-acetylated α-tubulin antibody. The head is oriented towards the top of each image (objective magnification: 20×). White arrowheads indicate abnormal anterior commissure formation. Scale bar, 50 μm. (B) Quantitative comparison showing the percentage of embryos displaying abnormal anterior commissure morphology across *cdk5*+/+, *cdk5*+/−, and *cdk5*−/− groups (black regions). Sample sizes: n = 25 (*cdk5*+/+), n = 30 (*cdk5*+/−), n = 16 (*cdk5*−/−).