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Knockdown of Cyclin-dependent Kinase 10 (*cdk10*) Gene Impairs Neural Progenitor Survival via Modulation of *raf1a* Gene Expression*

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Background: CDK10 is involved in cell proliferation; however, the function of CDK10 in cell development remains unclear.
Results: The knockdown of *cdk10* results in the apoptosis of neural progenitor cells (NPCs) through enhanced *raf1a* expression.
Conclusion: Zebrafish *cdk10* exhibits an important function in neurogenesis by the modulation of the *raf1a* level during development.
Significance: These findings emphasize the significance of *cdk10* in NPC survival.

In this study, we used zebrafish as an animal model to elucidate the developmental function of *cdk10* in vertebrates. *In situ* hybridization analyses demonstrated that *cdk10* is expressed throughout development with a relative enrichment in the brain in the late stages. Similar to its mammalian ortholog, cdk10 can interact with the transcription factor ETS2 and exhibit kinase activity by phosphorylating histone H1. Morpholino-based loss of *cdk10* expression caused apoptosis in *sox2*-positive cells and decreased the expression of subsequent neuronal markers. Acetylated tubulin staining revealed a significant reduction in the number of Rohon-Beard sensory neurons in *cdk10* morphants. This result is similar to that demonstrated by decreased *islet2* expression in the dorsal regions. Moreover, *cdk10* morphants exhibited a marked loss of *huC*-positive neurons in the telencephalon and throughout the spinal cord axis. The population of retinal ganglion cells was also diminished in *cdk10* morphants. These phenotypes were rescued by co-injection of *cdk10* mRNA. Interestingly, the knockdown of *cdk10* significantly elevated *raf1a* mRNA expression. Meanwhile, an MEK inhibitor (U0126) recovered *sox2* and *ngn1* transcript levels in *cdk10* morphants. Our findings provide the first functional characterization of *cdk10* in vertebrate development and reveal its critical function in neurogenesis by modulation of *raf1a* expression.

Protein kinase signals mediate various cellular functions such as metabolism, cell proliferation, cell growth, and apoptosis (1, 2). The cyclin-dependent kinase (CDK)[2] family of proteins is a group of serine/threonine kinases that regulate cell cycle progression specifically at the G_1/S and G_2/M checkpoints (3, 4). Cell cycle progression requires the association of CDK with specific cyclins to form active complexes, which subsequently control the expression of downstream cell cycle genes (3, 4).

A family of cdc2-related kinases, including PISSLRE (CDK10), PCTAIRE, KKIALRE (CDK-like-1; CDKL1), KKIAMRE (CDK-like-2; CDKL2), and NKIATRE (CDK-like-3; CDKL3), was recently identified based on their biochemical and genetic structures. Their names refer to their sequence, which corresponds to the cdc2 PSTAIRE motif (5). No known cyclins are associated with cdc2-related kinases. Northern blot analyses revealed that human CDK10 is broadly expressed in normal and tumor tissues (6). *In vitro* kinase assays also showed that CDK10 can phosphorylate histone H1 and myelin basic protein (6). ETS2 is a CDK10-interacting protein identified by yeast two-hybridization screening (7). CDK10 is associated with the N-terminal domain of ETS2, which harbors the transactivation domain and the highly conserved point domain of ETS2. However, CDK10 is not associated with ETS1 (7). When CDK10 binds to ETS2, CDK10 can inhibit the transactivation activity of ETS2 (7). Several reports have suggested that CDK10 is involved in cell proliferation and differentiation. In human U2OS cells, the abrogation of the CDK10 signal may be achieved by using a kinase-dead form of CDK10 or antisense RNA-arrested cell cycle progression at the G_2/M checkpoint (8). CDK10 was significantly down-regulated in retinoic acid-induced differentiation of retinoblastoma cells (9). Using two-dimensional gel electrophoresis and mass spectrometry analysis, Leman *et al.* (10) demonstrated the down-regulation of CDK10 in seminomas. CDK10 was shown to be up-regulated in follicular lymphomas compared with normal germinal center B-cells (11). CDK10 was also reportedly involved in endocrine therapy resistance in breast cancer (12). Using siRNA screening, Iorns *et al.* (12) found that changes in CDK10 expression can increase ETS2 transcriptional activity, elevate c-raf expression, and enhance MAPK activation, resulting in resistance to tamoxifen treatment.

Zebrafish is a widely used animal model in the study of genetic and developmental biology (13). To date, the role of

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[2] The abbreviations used are: CDK, cyclin-dependent kinase; MO, morpholino oligonucleotide; RB, Rohon-Beard; RGC, retinal ganglion cell; *nes*, *nestin*; CDKL, CDK-like; *ngn1*, *neurogenin-1*; *ef1α*, elongation factor 1α; HPF, h postfertilization; AcTub, acetylated α-tubulin; DPYSL, dihydropyrimidinase-like protein; 5-mis, 5-mismatch; *PCNA*, proliferating cell nuclear antigen.

CDKs in zebrafish development remains poorly understood. Zebrafish *cyclin H*/*cdk7* is ubiquitously expressed throughout all examined stages and tissues (14). Abrogation of *cyclin H* expression results in defective development and apoptosis (14). Previous reports revealed that zebrafish *cdk5* is ubiquitously and abundantly expressed in the brain and the eyes (15). Knockdown of zebrafish *cdk5* by using siRNA decreases the number of primary sensory Rohon-Beard (RB) neurons in the trigeminal ganglia and promotes apoptosis in the brain (15, 16). Meanwhile, morpholino-based knockdown of *cdk5* increases the number of supernumerary motor neurons (17). Recently, *cdkl1* was reported to fulfill a function in zebrafish development. Interruption of *cdkl1* expression decreases *neurogenin-1* (*ngn1*) levels in the brain and *sonic hedgehog* expression in the floor plate region (18). In the present study, we used zebrafish as an animal model to investigate the developmental role of *cdk10*.

EXPERIMENTAL PROCEDURES

Reagents—All of the chemical reagents were purchased from Sigma or Mallinckrodt Baker. Antibodies against human CDK10, human ETS2, and GFP and HRP-conjugated goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology. The anti-β-actin antibody was obtained from Sigma. Anti-sox2 antibody (ab97959) was purchased from Abcam.

Maintenance of Zebrafish—The zebrafish AB strain and transgenic *Tg* (*huC:gfp*) fish were kind gifts from the Taiwan Zebrafish Core Facility (Academia Sinica, Taiwan) and Dr. Chang-Jen Huang (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan), respectively. All of the fish were raised and maintained under standard conditions at 28.5 °C under a 14-h light/10-h dark cycle (19). The developmental stages of the embryos were determined according to methods described previously (20). All of the animal handling protocols were approved by the Chung Shan Medical University Animal Care Committee. Images of the fluorescent signals were captured using a Zeiss LSM510 confocal microscope.

RNA Extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Quantitative Real Time PCR Analysis, and Plasmid Constructs—Total RNA was isolated from different tissues and stages in adult zebrafish using TRIzol (Invitrogen). First strand cDNA was synthesized from 3 μg of total RNA by Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. The full coding region of *cdk10* was amplified by RT-PCR using zebrafish cDNA as a template and the following primer set: forward, 5′-ATGGAGAGCACAGCAGACAC-3′; reverse, 5′-GTCATACTTTGCTCCTCTTGG-3′. The PCR was performed under the following conditions: 94 °C, 30 s; 52 °C, 60 s; 72 °C, 60 s for 30 cycles. The amplified products were then ligated into a pGEM-T-Easy vector (Promega) and transformed into competent cells. The pEGFP-*cdk10* and pCS2-*cdk10* were constructed by enzyme digestion and ligated into pEGFP-C1 and pCS2, respectively. Quantitative real time PCR was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) with SYBR Green fluorescent label (Thermo Scientific). Primers for zebrafish *raf1a* (forward, 5′-AAGACATCAGCTCCTGCACA-3′; reverse, 5′-AGACGATCACTCTTTGAGGG-3′) were used. Gene expression levels were normalized to elongation factor 1α (*ef1*α) (forward, 5′-TGCCTTCGTCCCAATTTCAG-3′; reverse, 5′-TACCCTCCTTGCGCTCAATC-3′) and assessed using comparative C_T (40 cycles) according to the manufacturer's instructions (Applied Biosystems). The expression level was normalized to *ef1*α and calculated using the $\Delta\Delta C_T$ method.

TUNEL Assay—A TUNEL assay was performed according to the manufacturer's instructions (Promega). Briefly, the embryos (24 or 48 HPF) were fixed with 4% paraformaldehyde at 4 °C overnight, dehydrated by washing three times in methanol, and stored at −20 °C for at least 30 min. After rehydration, the embryos were transferred to tubes containing 100 μl of equilibration buffer and incubated at room temperature for 10 min. Next, the embryos were incubated with 100 μl of labeling solution at 37 °C for 1 h. After washing with 2× SSC buffer and phosphate-buffered saline (PBS), the TUNEL signals were examined using an upright fluorescence microscope (Zeiss AXioskop2).

Cell Culture and Transfection—Mouse neuroblastoma Neuro-2a and human embryonic kidney (HEK) 293 cells were obtained from ATCC and maintained in minimum essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2. Plasmid DNAs were transfected into Neuro-2a or HEK 293 cells using the Lipofectamine reagent (Invitrogen). Twenty-four hours after transfection, the cell lysates were collected.

Immunoblot Analysis and in Vitro Kinase Assay—Five hundred micrograms of total cell lysates derived from HEK 293 cells overexpressing GFP alone or the GFP-cdk10 fusion protein was subjected to immunoprecipitation using an anti-GFP antibody, and a kinase assay was performed as described previously (18, 21). After washing three times with kinase buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, and 0.1 mM sodium orthovanadate), the precipitates were incubated with H1 histone in kinase buffer supplemented with 0.1 mM ATP, pH 7.0, 5 μCi of [γ-^{32}P]ATP, and 10 mM $MgCl_2$ at 37 °C for 10 min. After separation by electrophoresis with a 12.5% SDS-polyacrylamide gel, the phosphorylation status was determined by autoradiography of the dried gel.

Microinjection of Antisense Morpholino Oligonucleotides (MOs) and Synthetic mRNA—The following MOs were designed and synthesized by Gene Tools, LLC (Philomath, OR): *cdk10* MO (5′-GGTCTGTGTCTGCTGTGCTCTCCAT-3′), which targets the start codon (from +1 to +25); 5-mismatch (5-mis) MO (5′-GGTGTCTGTCTCCTGTCCTGTCCAT-3′) where the underlines indicate the mismatched sites; and control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′), which corresponded to the human β-globin gene and was used as a negative control. MOs were dissolved in 1× Danieau solution and injected into the embryos at the one- to four-cell stage. The pCS2-*cdk10* plasmid was linearized by NotI, and the mRNA was synthesized by SP6 RNA polymerase using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's recommendation. For the rescue experiments, morpholinos were co-injected with 11 pg of mRNA into one- to four-cell embryos.

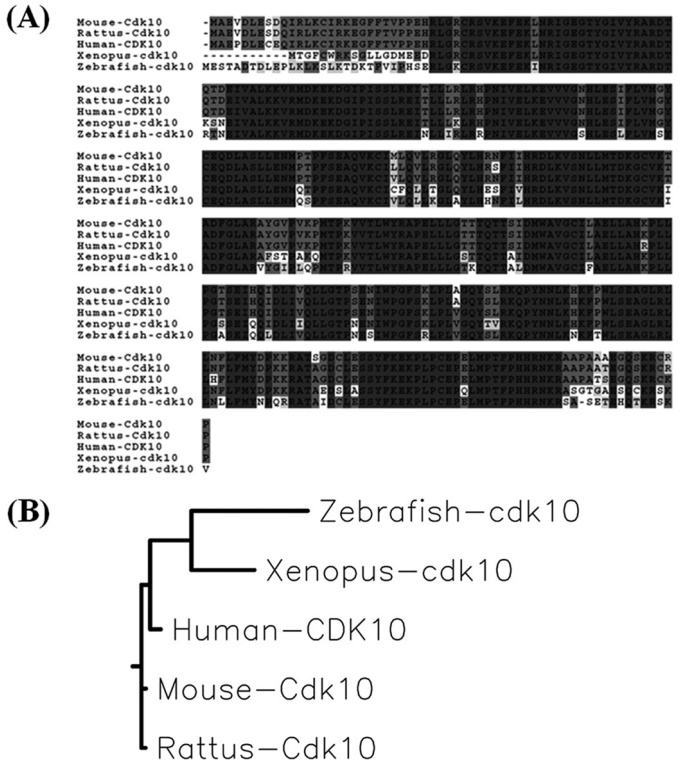


FIGURE 1. **Protein sequence alignments and phylogenetic tree.** *A*, sequence alignment of zebrafish *cdk10* with that of human, rat, mouse, and *Xenopus*. Identical amino acids in all or three species are shaded in *black* and *light gray*, respectively. *B*, phylogenetic tree of zebrafish *cdk10*. The phylogenetic tree was created using the MegAlign program in DNASTAR using the neighbor joining algorithm.

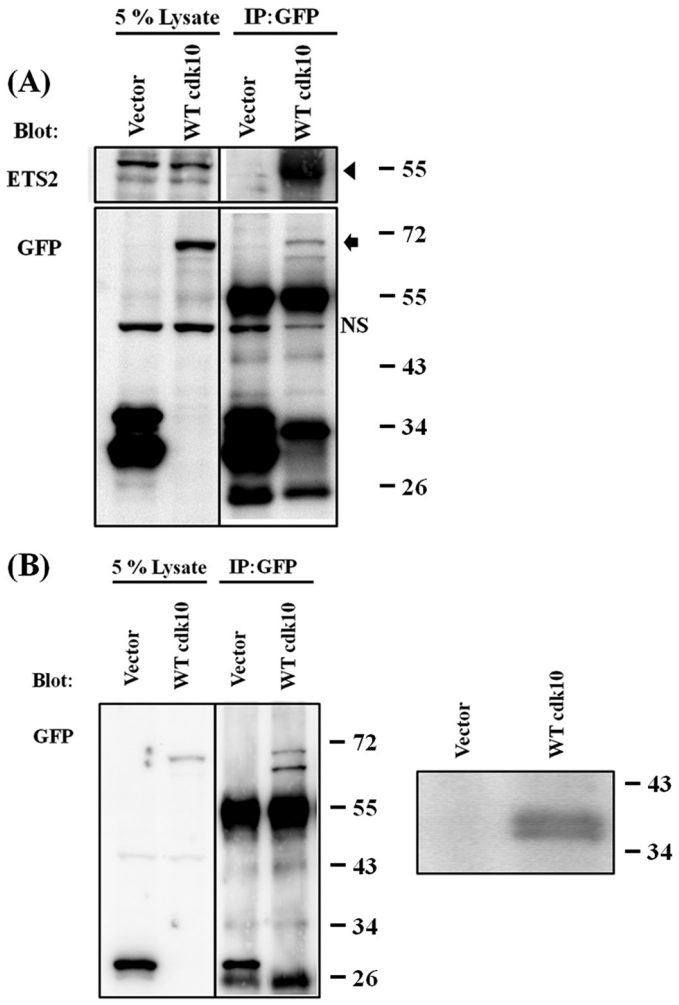


FIGURE 2. **Characterization of zebrafish *cdk10*.** *A*, zebrafish cdk10 interacts with ETS2. pEGFP or pEGFP-zcdk10 was transfected into HEK 293 cells. At 24 h post-transfection, cell lysates were collected and immunoprecipitated (*IP*) with an anti-GFP antibody. *NS* denotes nonspecific signal. The *arrowhead* indicates ETS2 signals. The *arrow* indicates GFP-cdk10 signals. *B*, *in vitro* kinase assay. pEGFP or pEGFP-*zcdk10* was transfected into HEK 293 cells. GFP fusion proteins were immunoprecipitated with an anti-GFP antibody and subjected to an *in vitro* kinase assay using histone H1 as substrate. *Left panel*, immunocomplexes were analyzed using Western blot and anti-GFP antibody. *Right panel*, phosphorylated signals were detected by autoradiography.

Whole-mount in Situ Hybridization—Whole-mount *in situ* hybridization was performed as described previously (22). Digoxigenin-labeled antisense riboprobes were generated from a pGEM-T-Easy plasmid containing the full coding sequence and partial 3′-untranslated regions of *cdk10*. The plasmid was linearized using NotI. The RNA was transcribed *in vitro* by the SP6 RNA polymerase to generate antisense probes using the DIG-RNA labeling kit (Roche Applied Science).

Western Blot Analysis—Zebrafish embryos injected with the control MO, *cdk10* MO, 5-mismatch MO, or *cdk10* MO plus *cdk10* mRNA were collected at 24 HPF. The total protein was extracted as described above. Thirty micrograms of protein was separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with PBS containing 5% nonfat milk, washed with PBS buffer, and incubated with anti-human CDK10 antibody (Santa Cruz Biotechnology) overnight. Next, the membranes were incubated with an HRP-conjugated goat anti-mouse IgG antibody, and the signals were detected using an enhanced chemiluminescence kit (Millipore).

Co-immunoprecipitation—HEK 293 cells were transfected with 2 μg of pEGFP-*cdk10* or pEGFP vector, and the cell lysates were collected as described above. Four hundred micrograms of proteins was subjected to immunoprecipitation using an anti-GFP antibody. The immunoprecipitated complexes were separated by electrophoresis on a 10% polyacrylamide gel followed by immunoblot analysis using anti-human ETS2 and anti-GFP antibodies. The signal was detected using an enhanced chemiluminescence kit (Millipore).

Anti-acetylated α-Tubulin Antibody Staining—Briefly, embryos at the indicated stages were collected, fixed in 4% paraformaldehyde at 4 °C overnight, and stored in methanol at −20 °C. After rehydration with PBST (PBS containing 0.1% Tween 20), the embryos were blocked with blocking solution (5% goat serum, 2% bovine serum albumin, and 1% DMSO in PBST) for 1 h and then incubated with monoclonal anti-acetylated α-tubulin at 4 °C overnight. Samples were washed with PBST and incubated with goat anti-mouse FITC-conjugated antibodies (1:500) in blocking buffer at 4 °C overnight in the dark. After washed with washing buffer (2% BSA and 1% DMSO in PBST), the images were captured by a scanning laser confocal microscope (Zeiss 510 META).

Statistical Analysis—Statistical analysis was performed by Student's *t* test using SPSS software (SPSS, Inc.). A p value <0.05 was considered statistically significant.

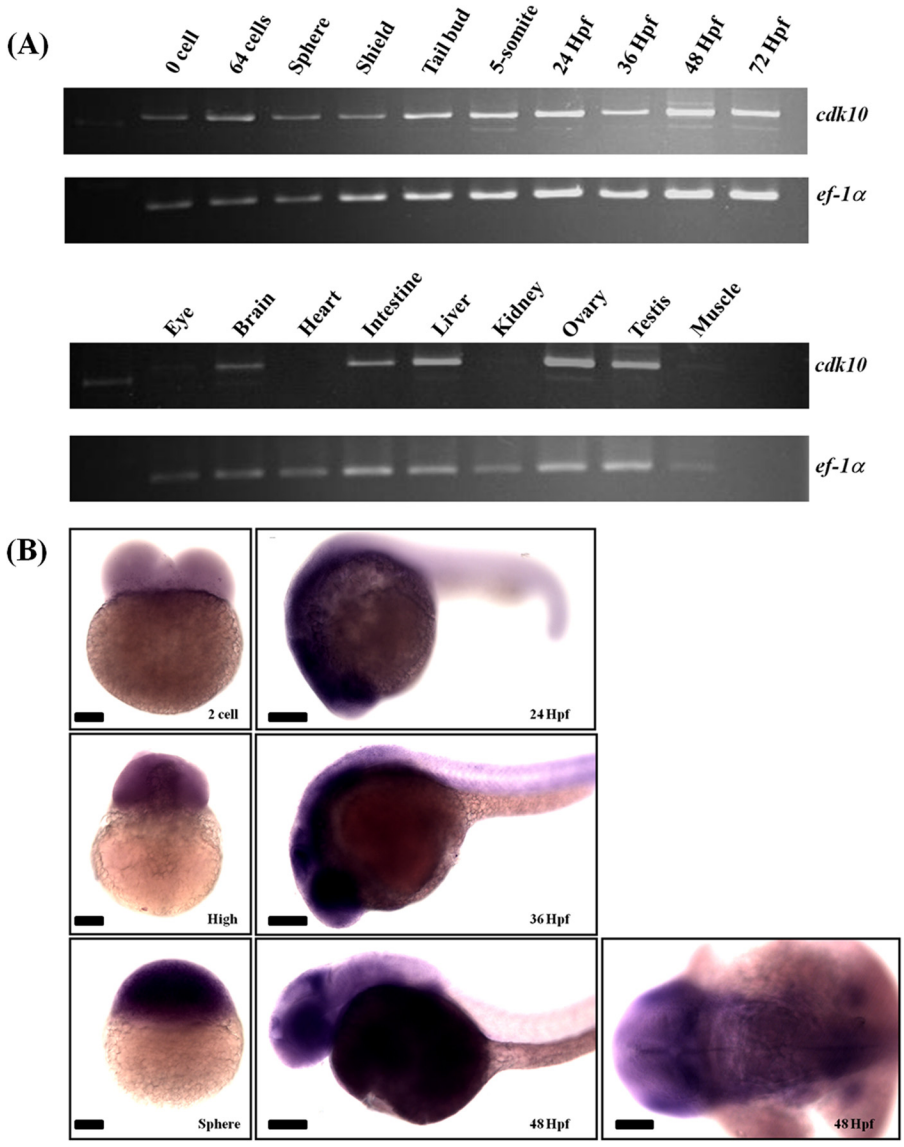


FIGURE 3. **Spatial and temporal expression pattern of zebrafish *cdk10*.** *A*, RT-PCR analyses of *cdk10* mRNA in different stages (*upper panel*) and different adult tissues (*lower panel*). Elongation factor 1α is used as internal control. *B*, whole-mount *in situ* hybridization analyses of *cdk10* mRNA during zebrafish development. Zebrafish *cdk10* is expressed both maternally and zygotically throughout development with relative enrichment in the brain. Stages are indicated in the *lower right*. *Scale bars*, 100 μm.

RESULTS

Identification of Zebrafish cdk10—We performed Basic Local Alignment Search Tool program analysis using the human CDK10 protein sequence to study the function of *cdk10* in zebrafish. A gene (NCBI Reference Sequence accession number NM_001017622) that encodes the full-length zebrafish *cdk10* was identified. Zebrafish *cdk10* contains 1298 base pairs; encodes a polypeptide of 360 amino acid residues; and displays 78, 77, 77, and 76% identity to human, mouse, rat, and *Xenopus* orthologs, respectively. The kinase domain extended from residues 48 to 300, and the conserved PISSLRE motif was localized at amino acids 81–87 (Fig. 1*A*). On the basis of the phylogenetic analysis, zebrafish *cdk10* was closer to *Xenopus cdk10* (Fig. 1*B*).

Functional Characterization of cdk10—A previous report showed that human CDK10 interacts with the transcription factor ETS2 (7). We performed a co-immunoprecipitation assay to investigate whether cdk10 interacts with human ETS2. A strong ETS2 signal was detected in the complexes immunoprecipitated with GFP-cdk10 but not in those immunoprecipitated solely with GFP (Fig. 2*A*). These results demonstrated that cdk10 interacts with ETS2 and are similar to the results obtained with human and murine CDK10.

We performed an *in vitro* kinase assay to confirm whether cdk10 exhibits kinase activity. The GFP-cdk10 fusion protein was overexpressed in the HEK 293 cell line and purified with an anti-GFP antibody. Histone H1 was phosphorylated by cdk10 in the presence of an isotope-labeled ATP (Fig. 2*B*). Similar to its mammalian ortholog, cdk10 was shown to interact with ETS2 and exhibit kinase activity during the phosphorylation of histone H1.

Temporal and Spatial Expression Patterns of cdk10—We conducted RT-PCR and whole-mount *in situ* hybridization to analyze the developmental expression patterns of *cdk10*. RT-

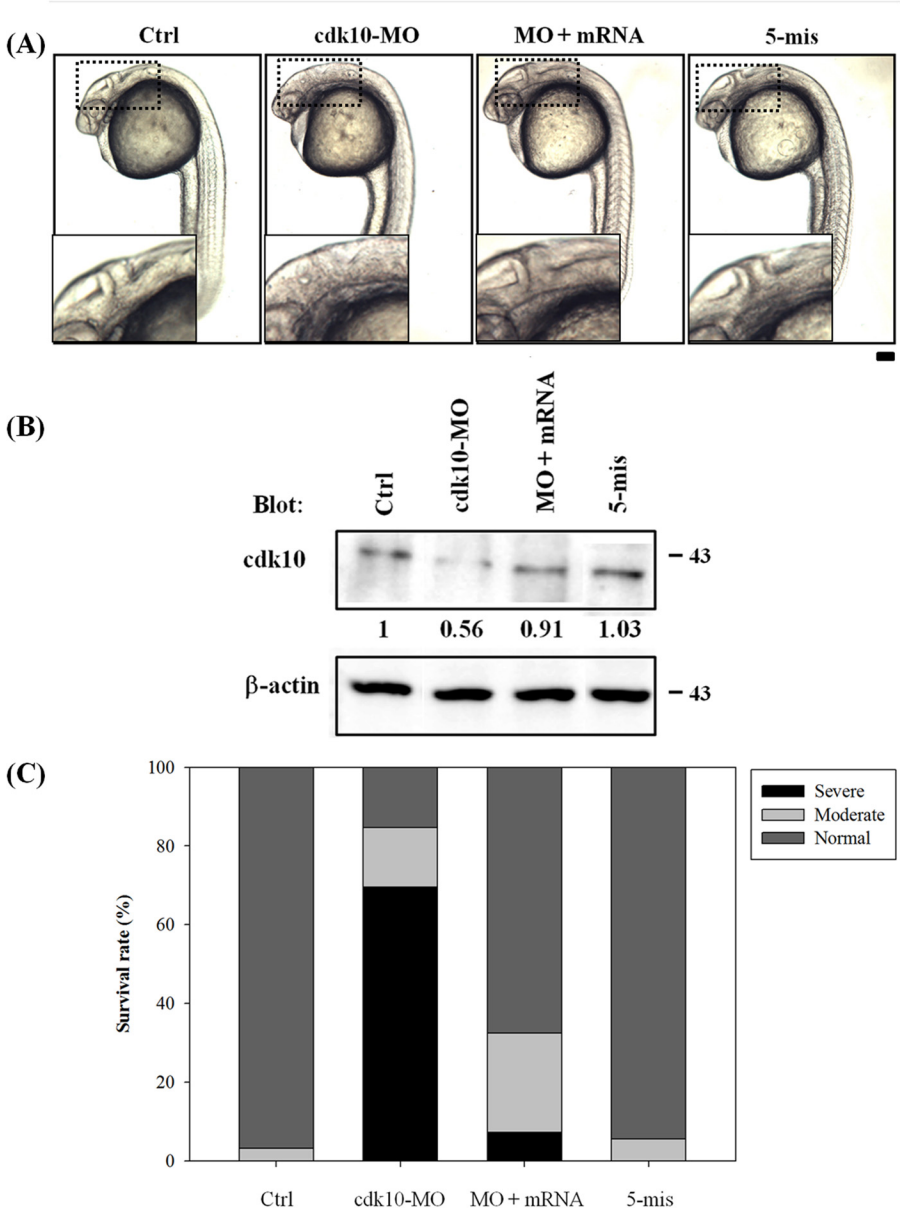


FIGURE 4. **Morphological phenotype of *cdk10* morphants.** *A*, phenotypic analyses of zebrafish embryos at 24 HPF. Embryos at one- to four-cell stages that received 1 ng of control MO (*Ctrl*), 1 ng of *cdk10* MO, 1 ng of *cdk10* MO plus 10 pg of *cdk10* mRNA (*MO + mRNA*), and 1 ng of 5-mismatch *cdk10* MO (*5-mis*) are depicted at 24 HPF. Compared with the normal morphology of the wild type (WT), *cdk10* morphants exhibited head malformations with indistinct boundaries between the brain subdivisions as shown in the small *insets*. Images are shown in the lateral view with the anterior at the *left*. *B*, Western blot analysis of *cdk10* knockdown efficiency. Embryos injected with the indicated MO were harvested at 24 HPF. Protein samples were prepared and probed using a human CDK10-specific antibody. β-Actin was used as the loading control. Data represent one of at least three independent experiments. *C*, percentages of normal, moderate, and severe phenotypes in the WT control ($n = 159$), *cdk10* MO ($n = 193$), MO + mRNA ($n = 279$), and 5-mis MO ($n = 178$) embryos. *Scale bar*, 100 μm.

PCR analysis showed that *cdk10* was expressed in all examined zebrafish stages. Furthermore, *cdk10* was predominantly expressed in the adult brain, intestine, liver, ovary, and testis. Whole-mount *in situ* hybridization revealed that *cdk10* mRNA was ubiquitously expressed from the two-cell to sphere stages. At 24 and 36 HPF, *cdk10* expression was relatively enriched in the eye and brain regions. At 48 HPF, *cdk10* was predominantly expressed in the eye, tectum, hindbrain-midbrain boundary, and telencephalon (Fig. 3, *A* and *B*).

Developmental Role of cdk10—Antisense MOs that overlap with the translation start codon were designed to block the translation of the *cdk10* gene and thereby explain the function of *cdk10* during zebrafish development. To confirm whether *cdk10* MO affects endogenous *cdk10* expression, protein extracts from embryos receiving control MO, *cdk10* MO, and 5-mis MO were subjected to Western blot analysis using anti-human CDK10 antibody. As shown in Fig. 4*B*, 1 ng of *cdk10* MO reduced the amount of cdk10 protein expression by 44%. However, no clear changes in cdk10 expression were observed in embryos that received control MO or 5-mis MO. These results indicated that *cdk10* MO specifically blocks the translation of the endogenous *cdk10* gene. A previous report found that the microinjection of specific MOs induces off-target effects by increasing p53 activity, resulting in cell death (23). These off-target effects were avoided in this study by using specific MOs that were co-injected with 2-fold amounts of *p53* MO in all MO

experiments. To determine whether *p53* MO contributes to the phenotype of *cdk10* morphants, we injected the embryos with *p53* MO alone. Indeed, no distinct phenotype was observed between the *p53* morphants and the control embryos (data not shown). At 24 HPF, embryos receiving 1 ng of *cdk10* MO exhibited significant developmental abnormalities such as small brain, undersized eye, and somite curvature. These embryos also lacked the isthmus region. The percentages of normal, moderate, and severe phenotypes (characterized by smaller brain size, undersized eyes, and curved somites) are presented in Fig. 4*C*. Developmental defects observed in *cdk10* morphants as well as endogenous cdk10 protein level could be rescued by co-injection of *cdk10* mRNA, demonstrating the specificity of this morpholino (Fig. 4, *A* and *B*). Dark cell masses observed in *cdk10*-depleted embryos indicated that these morphants underwent cell death. To verify this hypothesis, we performed a TUNEL assay to examine the embryos for apoptosis. Compared with the control MO-injected embryos, depletion of *cdk10* sharply increased the number of TUNEL-positive cells in the dorsal neuronal regions at the three-somite stage (in the brain at 24 HPF as well as the forebrain and the midbrain regions at 48 HPF). Co-injection with *cdk10* mRNA significantly reduced apoptosis (Fig. 5, *A*–*C*). These findings show the significance of *cdk10* during development in zebrafish.

Knockdown of cdk10 Abrogates Several Central Nervous System (CNS) Marker Expression—We examined the expression of *sox2* (a known neural stem cell marker) and *ngn1* (a marker of differentiating neurons at the three-somite stage) to evaluate the effect of *cdk10* on neural development. Depletion of *cdk10* resulted in expansion and reduction of *sox2* expression compared with wild-type control embryos (Fig. 6*A*). Loss of *cdk10* reduced *ngn1* expression in dorsal neuron precursor cells (Fig. 6*B*). At 24 HPF, morphant embryos showed a significant disruption of *ngn1* expression in the forebrain and the cranial ganglia (Fig. 6*C*). Significant reductions in *zath3* (a late proneural gene) and *nes* (a marker for stem cells) were observed in *cdk10* morphants (Fig. 6*D*). In addition, *wnt1* (alar plate midbrain region) expression was significantly reduced in the dorsal midline neurons of *cdk10*-depleted morphants at 24 HPF (Fig. 6*E*). We subsequently determined whether forebrain patterning events were disrupted in *cdk10* mutants. The expression of *emx1* (a marker involved in telencephalic patterning) was markedly reduced in *cdk10* morphants (Fig. 6*F*). The expression of *islet2* (a dorsal root ganglion marker) was significantly reduced in RB neurons, whereas motor neurons were not affected in *cdk10* morphants (Fig. 6*G*). These findings suggest the significance of *cdk10* in the formation of forebrain and midbrain as well as RB neurons.

To determine the role of *cdk10* during neurodevelopment, we conducted acetylated α-tubulin (AcTub) staining to determine whether disruption of *cdk10* affects neuronal differentiation. At 24 HPF, AcTub staining revealed the presence of anterior commissure, postoptic commissure, supraoptic tract, nucleus of the tract of anterior commissure, dorsal-ventral diencephalic tract, epiphyseal cluster, and tract of the posterior commissure in the forebrain and the midbrain in the embryos injected with control and 5-mis MOs (Fig. 7*A*). Axonal scaffolds in *cdk10* morphants visibly deteriorated. Reduced arborization

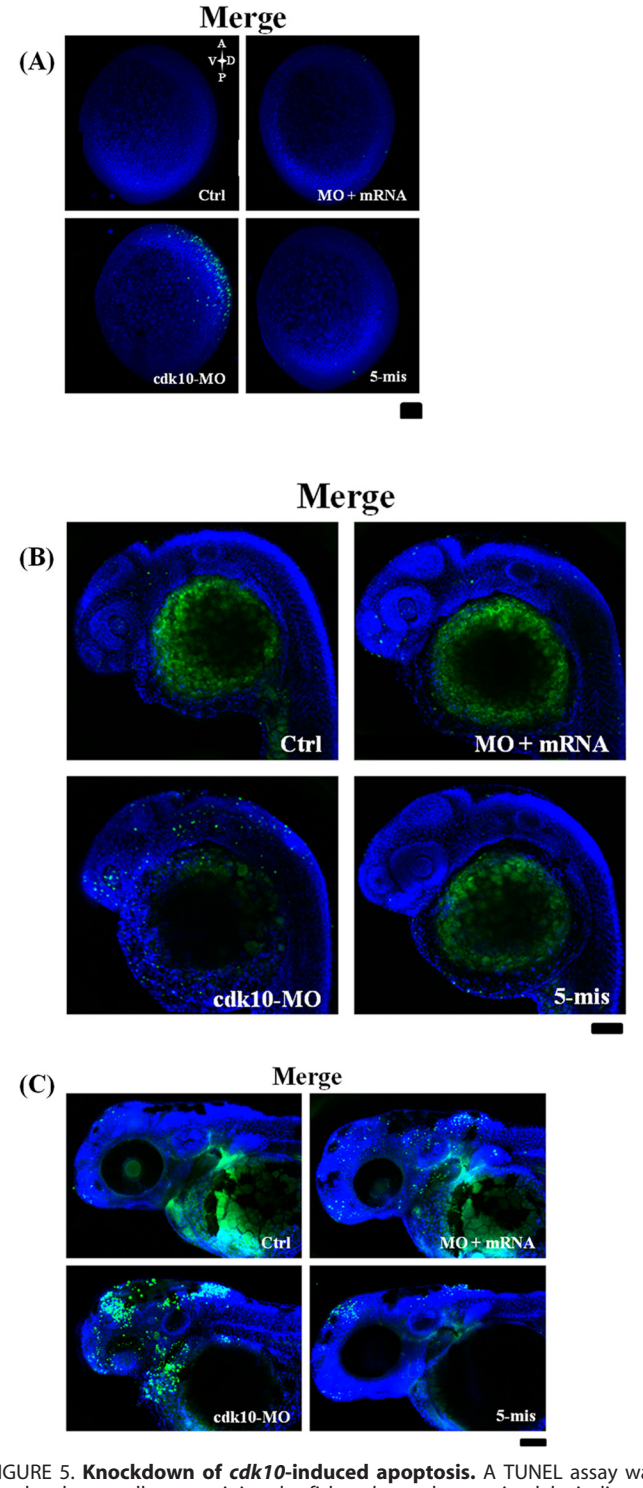


FIGURE 5. **Knockdown of *cdk10*-induced apoptosis.** A TUNEL assay was used to detect cell apoptosis in zebrafish embryos that received the indicated MO in the tail bud stage (*A*), at 24 HPF (*B*), or at 48 HPF (*C*). Control MO (*Ctrl*)-injected embryos displayed very few TUNEL-positive cells. The *cdk10* MO-injected embryos that were primarily concentrated in the forebrain, midbrain, neural plate, and retina regions showed a high level of apoptosis. Apoptosis was prevented by co-injection of *cdk10* mRNA. All images are shown in the lateral view with the anterior at the *left* ($n = 10$ embryos each). *Scale bars*, 100 μm. *A*, anterior; *V*, ventral; *D*, dorsal; *P*, posterior.

was evident in several neurons, including those in the spinal cord (Fig. 7*B*). Embryos co-injected with *cdk10* mRNA resembled those of the control group. Similar results were also observed at 48 HPF at which time the staining patterns of the

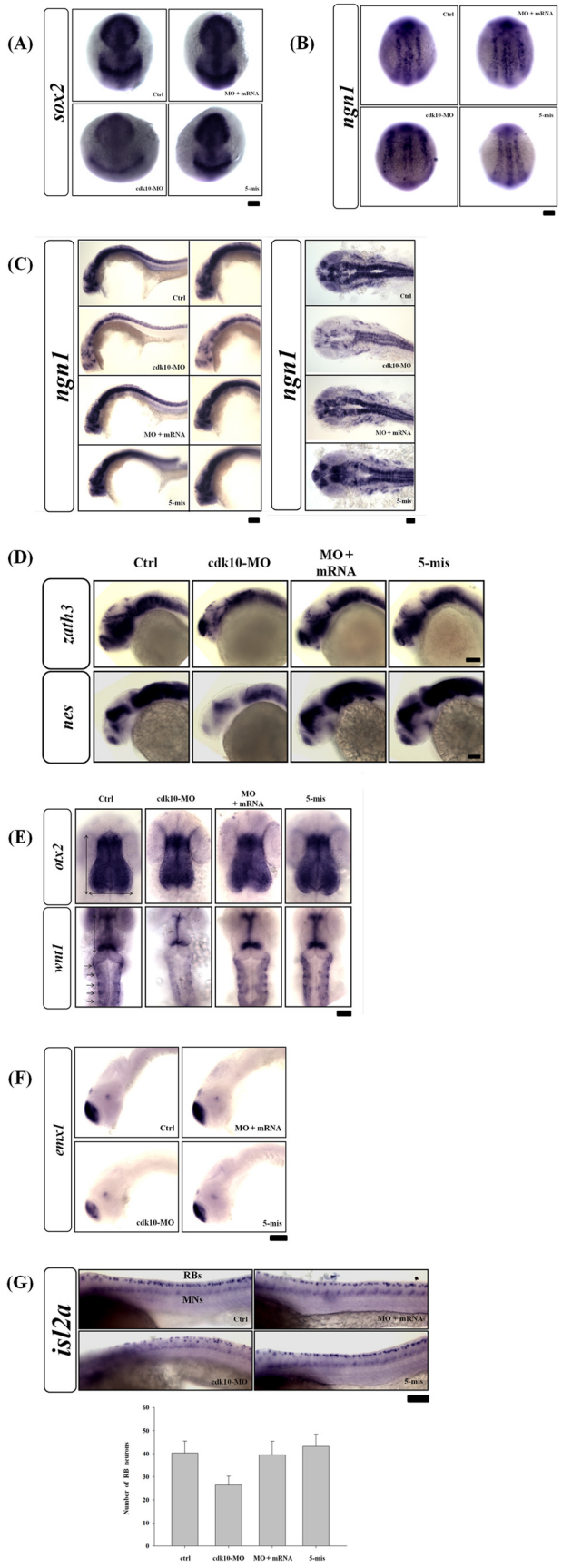


FIGURE 6. **Knockdown of *cdk10*-impaired zebrafish neuronal development and affected RB sensory neuronal population.** In preparation for whole-mount *in situ* hybridization with specific neuronal markers, embryos that received the indicated MO with or without corresponding RNA were collected at the tail bud stage (*A* and *B*) and at 24 HPF (*C*–*F*). *A* and *B*, depletion of *cdk10* induced a local decrease and expansion of the neural plate markers *sox2* and *ngn1*. *C*, the *cdk10* morphants significantly disrupted the *ngn1* expression in the CNS (*right and middle panels*, lateral view; *left panel*, dorsal view). *D*, lateral views of whole-mount *in situ* hybridization detecting *zath3* and *nes* in embryos at 24 HPF. Expression of *zath3* and *nes* was altered in *cdk10* morphants. These defects can be complemented by co-injection of *cdk10* mRNA. *E*, loss of *wnt1* expression in dorsal midline neurons (denoted with an *arrow*) was found in *cdk10* morphants, whereas no apparent change in *otx2* expression was observed (dorsal view). *F*, the expression of the telencephalic marker *emx1* was reduced in *cdk10* morphants (lateral view). *G*, the number of RB neurons (*RBs*) was decreased in *cdk10* morphants as shown by the decrease in *islet2* expression. Conversely, the number of motor neuron cells (*MNs*) was not affected in *cdk10* morphants. *Lower panel*, quantitative analyses of *islet2*-positive RB neurons. Data were obtained from at least three independent experiments. *Scale bars*, 100 μm. *Ctrl*, control. *Error bars* represent S.D.

tectal neurons, hindbrain neurons, and tubulin-labeled axons were severely reduced in *cdk10* morphants. Co-injection with specific *cdk10* mRNA restored these defects (Fig. 7*C*). Therefore, depletion of *cdk10* caused abnormalities in the number of neurons and in axonal pathfinding, indicating that *cdk10* is involved in the proper maintenance of neurons.

We also used the *Tg* (*huC:gfp*) model to track the generation of terminally differentiated neurons. A lateral view of the embryos at 24 HPF revealed that the control, 5-mis MO, and *cdk10* MO plus *cdk10* mRNA groups exhibited high levels of *huC* expression in the telencephalic cluster and neural tube. We observed that *cdk10* morphants exhibited abrogated *huC* expression in the forebrain region and a significantly decreased *huC* population in the neural tube throughout the spinal cord axis (Fig. 8, *A* and *B*). At 48 HPF, only a small number of *huC*-positive neurons were present in the tectum and hindbrain regions of *cdk10* morphants (Fig. 8*C*). A dorsal view of the embryos revealed that *cdk10* morphants also exhibited severe defects of neuronal organization in the olfactory bulb, telencephalic cluster, and retinal ganglion (Fig. 8*D*). Thus, *cdk10* knockdown in zebrafish embryos affects neuronal development in the brain.

Zebrafish cdk10 Did Not Directly Contribute to the Cell Cycle—The results showed that *cdk10* knockdown induced cell apoptosis (Fig. 5) and hindered CNS development (Figs. 7 and 8). This phenomenon of cell death may result in compensatory proliferation, thereby influencing cell cycle distribution and abrogation of differentiation. This finding is consistent with the results of previous studies on *Drosophila* (24), chicken (25), and zebrafish embryos (26). We then performed *in vitro* and *in vivo* assays to assess cell cycle progression using gain and loss of cdk10 function. HEK 293 cells with or without *cdk10* overexpression were collected and then analyzed for populations of cell cycle by flow cytometry. Our findings revealed no apparent change in cell cycle distribution in the cells transfected with the control or *cdk10* (Fig. 9*A*). Despite the *cdk10* knockdown, the transcript levels of cdk inhibitors such as *cdkn1b*, *cdkn1c*, *ccnd1*, and *PCNA* (a proliferation marker), were similar to the control in the CNS region (Fig. 9*B*). This result indicates that zebrafish *cdk10* did not elicit apoptosis-associated compensatory proliferation.

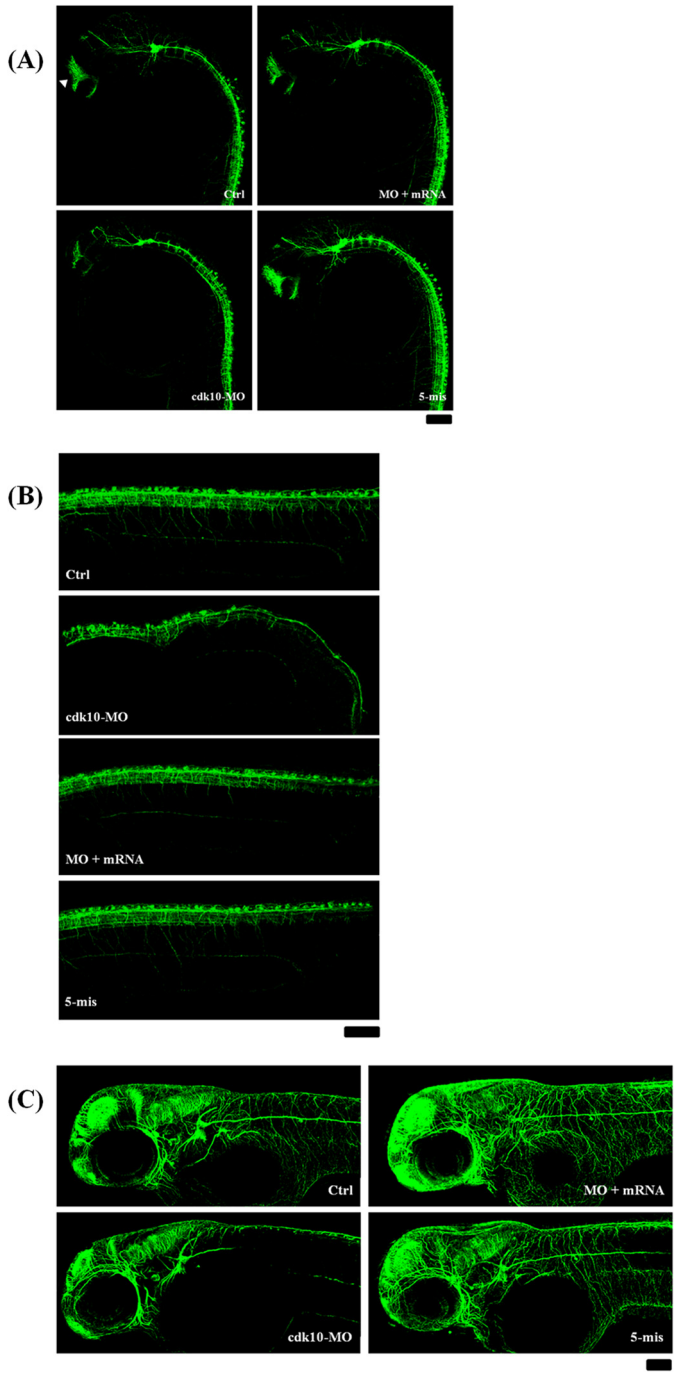


FIGURE 7. **Effects of *cdk10* depletion on neurogenesis as visualized by AcTub staining.** AcTub expression (an axonal marker) was examined using confocal microscopy at 24 (*A* and *B*) and 48 HPF (*C*). *A*, axonal scaffolds in *cdk10* morphants were visibly deteriorated. The *arrowhead* indicates anterior commissure. *B*, a decrease in the number of dorsal neurons, including RB neurons, was observed in *cdk10* morphants. *C*, the staining patterns of the tectal neurons, hindbrain neurons, and tubulin-labeled motor axons were severely reduced in *cdk10* morphants at 48 HPF. All images are shown in the lateral view. *Scale bars*, 100 μm. *Ctrl*, control.

Decrease in Differentiation of Retinal Cells in cdk10 Morphants—Using the *Tg* (*huC:gfp*) model, we observed fewer retinal ganglion cells (RGCs) in *cdk10* MO-injected embryos. We analyzed the expression of *zath5*, which is expressed in a wavelike fashion in the inner retina before the retinal ganglion cell differentiation (27). The expression of *zath5* in the control retina was found around the retina to the ventrotemporal region at 36 HPF, whereas the *zath5* signal was observed in differentiated RGCs near the ciliary region in normal retinas at 48 HPF. However, the spread of *zath5* expression from the ventral to the dorsal region was significantly repressed at 36 HPF. Although retinas of *cdk10* morphants seemed to have high levels of *zath5* expression, most *zath5*-expressing cells were located adjacent to the optic stalk even at 48 HPF. Embryos co-injected with *cdk10* mRNA resembled those of the control group. In addition, *brn3*, which is the downstream factor of *zath5* (28), exhibited severely diminished expression in the ganglion cell layer (Fig. 10, *A* and *B*). This result indicates that the onset of *zath5* expression was not delayed. However, fewer neurons were subsequently generated. Thus, *cdk10* is required during RGC production.

Zebrafish cdk10 Protects Neural Progenitor Survival—We evaluated whether the apoptotic cells observed in *cdk10* morphants were NPCs. We collected embryos at 80% epiboly and conducted immunostaining with fluorescent *sox2* antibody prior to TUNEL assay. In both early and late stages, most apoptotic cells were co-localized with *sox2*-positive cells in the anterior neural plate and throughout the axis of CNS in *cdk10* morphants. Co-injection with specific *cdk10* mRNA evidently reduced apoptosis, indicating that the apoptotic cells in *cdk10* morphants are NPCs (Fig. 11, *A* and *B*). By contrast, *p53* morphants presented no obvious TUNEL-positive signals in the early stages. These results show that the abrogation of *cdk10* may cause severe damage during NPC maintenance and subsequently disrupted neurogenesis.

raf1a Is Involved in cdk10-dependent Neuron Progenitor Survival—Previous studies reported that loss of CDK10 induces ETS2-dependent *RAF1* gene expression, thereby activating the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (12). This finding suggests that the zebrafish *raf1a* ortholog is also regulated by *cdk10*. To verify this hypothesis, we analyzed the transcriptional level of *raf1a* in *cdk10*-deficient embryos. The depletion of *cdk10* markedly elevated the transcription of *raf1a* (Fig. 12*A*). Co-injection with *cdk10* mRNA reduced *raf1a* expression to the basal level. No apparent alternation of *raf1a* expression was found in the embryos that received 5-mis MO. Embryos were co-treated with the MEK inhibitor U0126 in the presence of *cdk10* MO, and the expression of *sox2* and *ngn1* were determined to assess whether the *raf1a* involved in *cdk10* depletion induced NPC loss and aberrant neural differentiation. The knockdown of *cdk10* sharply decreased *sox2* expression at 80% epiboly. In addition, *ngn1* expression was significantly lower compared with the control at the tail bud stage and 24 HPF. Co-injecting *cdk10* mRNA restored *sox2* and *ngn1* expression. Interestingly, treatment with U0126 in *cdk10* morphants recovered *sox2* and *ngn1* expression to the same level as that of the control groups. By contrast, treatment with U0126 alone reduced *sox2* and *ngn1* expression, suggesting that basal levels of ERK1/2 activities were still required for normal neurogenesis (Fig. 12*B*). Our collective findings revealed that *raf1a* rescued the impaired NPC development caused by *cdk10* deficiency.

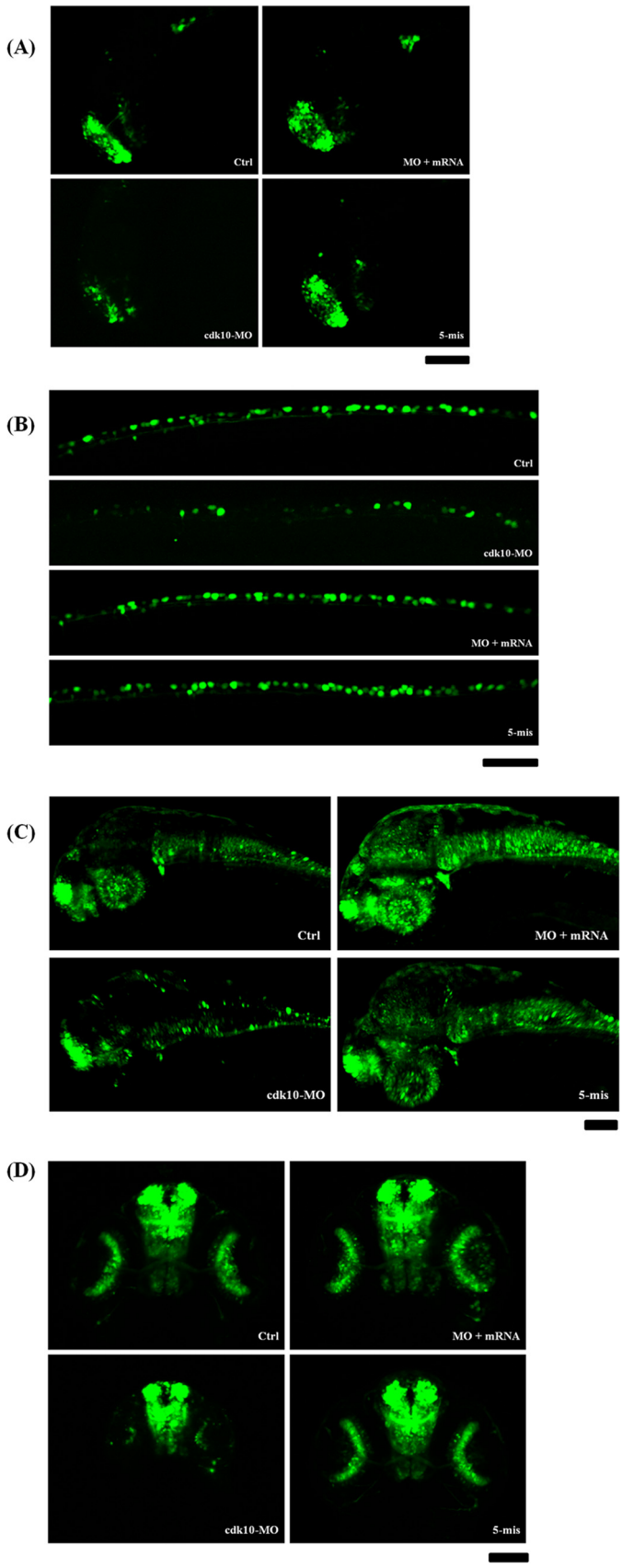
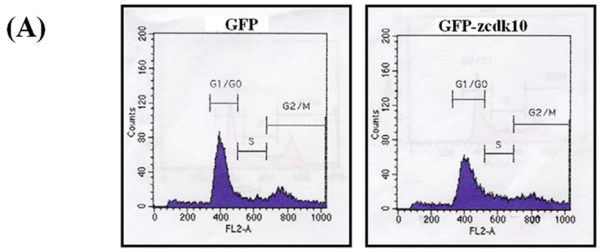


FIGURE 8. **Effects of *cdk10* depletion on neurogenesis on the basis of the *Tg* (*huC:gfp*) model.** Embryos that received the indicated MO were collected at 24 (*A* and *B*) and 48 HPF (*C* and *D*). *A*, the *huC* expression in the telencephalon decreased in *cdk10* morphants. Reduced numbers of RB neurons were also observed in *cdk10* morphants. *C* and *D*, the *cdk10* morphants exhibited abrogated *huC* expression in the forebrain region and a significantly decreased *huC* population of neurons in the retina. *A–C*, lateral view. *Scale bars*, 100 μm. *Ctrl*, control.



	GFP	GFP-zcdk10
G_0/G_1	42.5 ± 1.5 %	43.0 ± 2.1 %
S	15.3 ± 2.3 %	16.3 ± 1.5 %
G_2/M	21.7 ± 5.9 %	21.5 ± 6.1 %

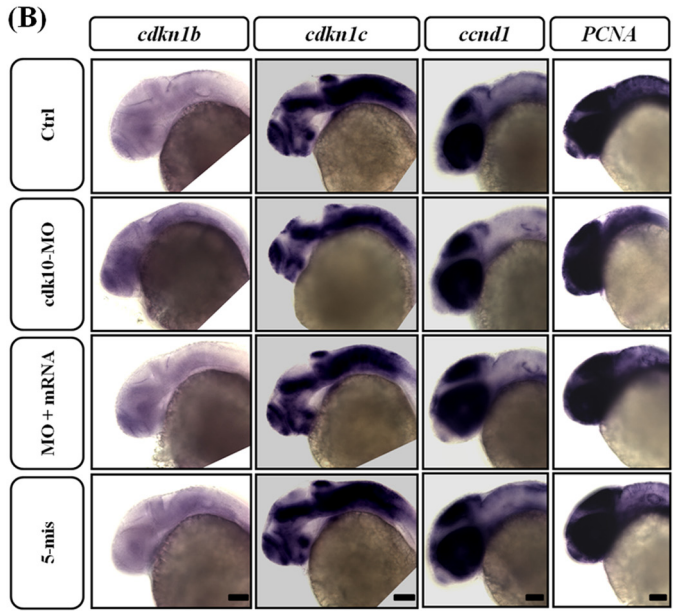


FIGURE 9. **Zebrafish *cdk10* did not participate in cell cycle progression *in vitro* and *in vivo*.** *A*, HEK 293 cell were transfected with pEGFP or pEGFP-cdk10. At 24 h post-transfection, cells were collected and subjected to flow cytometric analysis. The *lower panel* indicates the percentages of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle. *B*, lateral views of *cdkn1b*, *cdkn1c*, *ccnd1*, and *PCNA* expression in embryos injected with control (*Ctrl*), *cdk10* MO, MO + mRNA, and 5-mis MO at 24 HPF. Knockdown of *cdk10* showed no apparent difference in *cdkn1b*, *cdkn1c*, *ccnd1*, and *PCNA* mRNA transcription, suggesting that *cdk10* deficiency exhibited no influence on the progression of the cell cycle. *Scale bars*, 100 μm.

DISCUSSION

CDK10 is a serine/threonine protein kinase from the CDK family (5). Recent studies suggested that human CDK10 functions as a tumor suppressor gene, preventing cell proliferation in breast cancer (12), biliary tract cancer cells (29), and hepatoma (30). However, the developmental function of CDK10 remains unclear. We present evidence that zebrafish *cdk10* is involved in NPC survival. The disruption of *cdk10* expression by morpholinos caused defects in the brain by triggering apo-

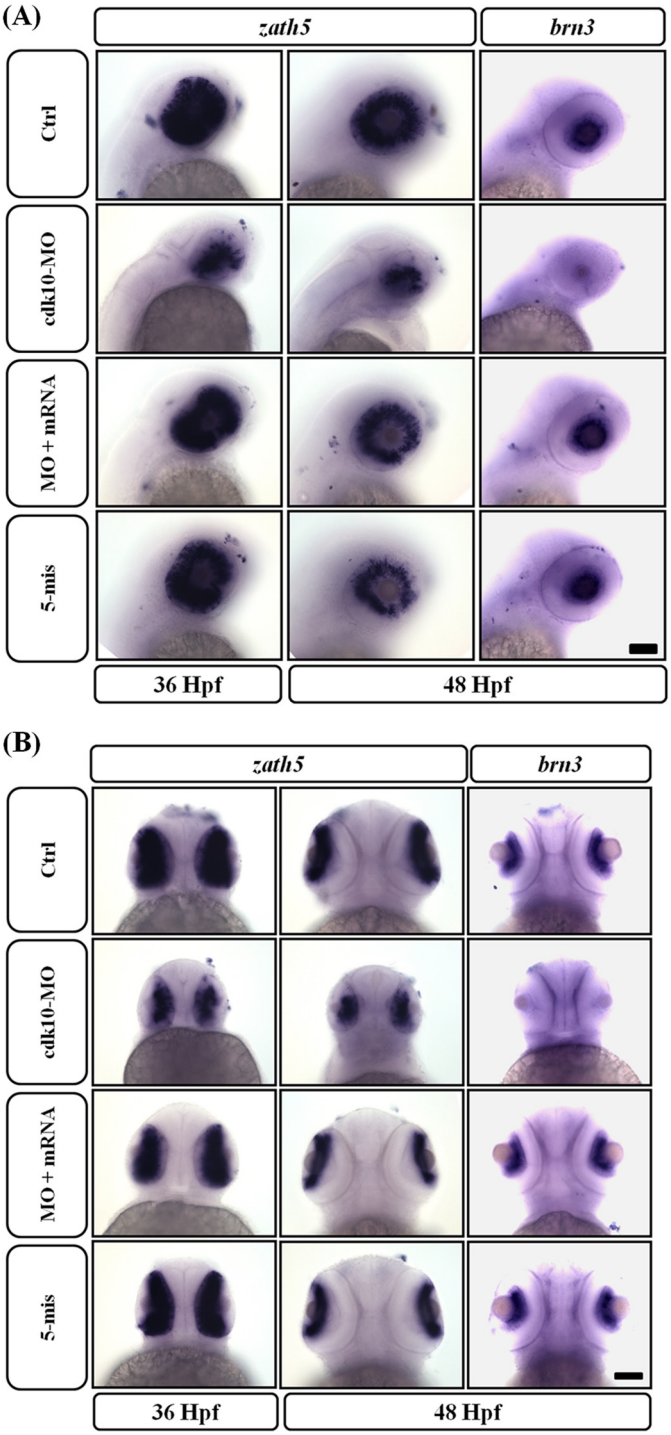


FIGURE 10. **Changes in *cdk10* levels impaired RGC differentiation.** *A* and *B*, expression of *zath5* (36 and 48 HPF) and *brn3* (48 HPF) mRNAs was examined by whole-mount *in situ* hybridization. At 36 HPF, *zath5* is undetected except in a small number of cells in *cdk10*-deficit embryos. At 48 HPF, the ciliary margin strongly expressed the *zath5* signal in the control retina, whereas the *zath5* signal is expressed near the optic stalk in the *cdk10* morphant retina. The expression of the downstream effector *brn3* is reduced severely at 48 HPF. *Scale bars*, 100 μm. *Ctrl*, control.

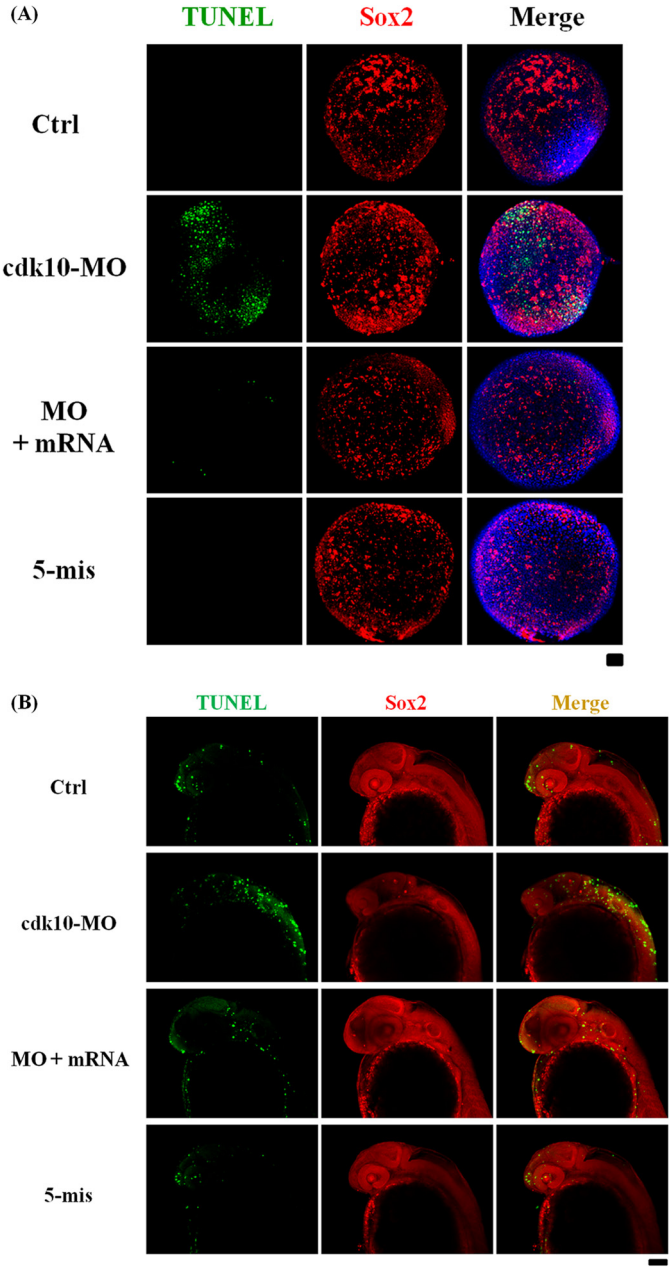


FIGURE 11. **Depletion of *cdk10* induced NPC apoptosis.** Fluorescent images of TUNEL staining (in *green*) and *sox2* expression (in *red*) in control (*Ctrl*), *cdk10* MO, MO + mRNA, and 5-mis MO in embryos at 80% epiboly (*A*) and 24 HPF (*B*). TUNEL- and *sox2*-positive cells were co-localized (in *yellow*), representing apoptotic NPCs ($n = 20$ embryos each). *Scale bars*, 100 μm.

ptosis in the brain and the dorsal neurons at 24 and 48 HPF, respectively. Whole-mount *in situ* hybridization revealed the down-regulation of several neuronal markers such as *ngn1*, *emx1*, *wnt1*, and *islet2* in morphants. Co-injection with *cdk10* mRNA rescued these phenotypes. Acetylated tubulin staining and *Tg* (*huC:gfp*) zebrafish demonstrated that the disruption of *cdk10* expression alters telencephalic patterns and decreases RB neurons and RGCs. The results of this study show that *cdk10* is an essential gene for zebrafish during normal embryogenesis.

The *cdk10* mRNA transcripts were diffusedly expressed in the early stages (prior to the sphere stage) and more abundant in the brain at 24 and 48 HPF. The progressively restricted pattern of *cdk10* expression was similar to that of other *cdk* and *cdk*-related genes in zebrafish. The *cdk7/cyclin H* and *cdkl1* genes are ubiquitously distributed before gastrulation (14, 18). At 24 HPF and thereafter, *cdk7* and *cyclin H* are primarily

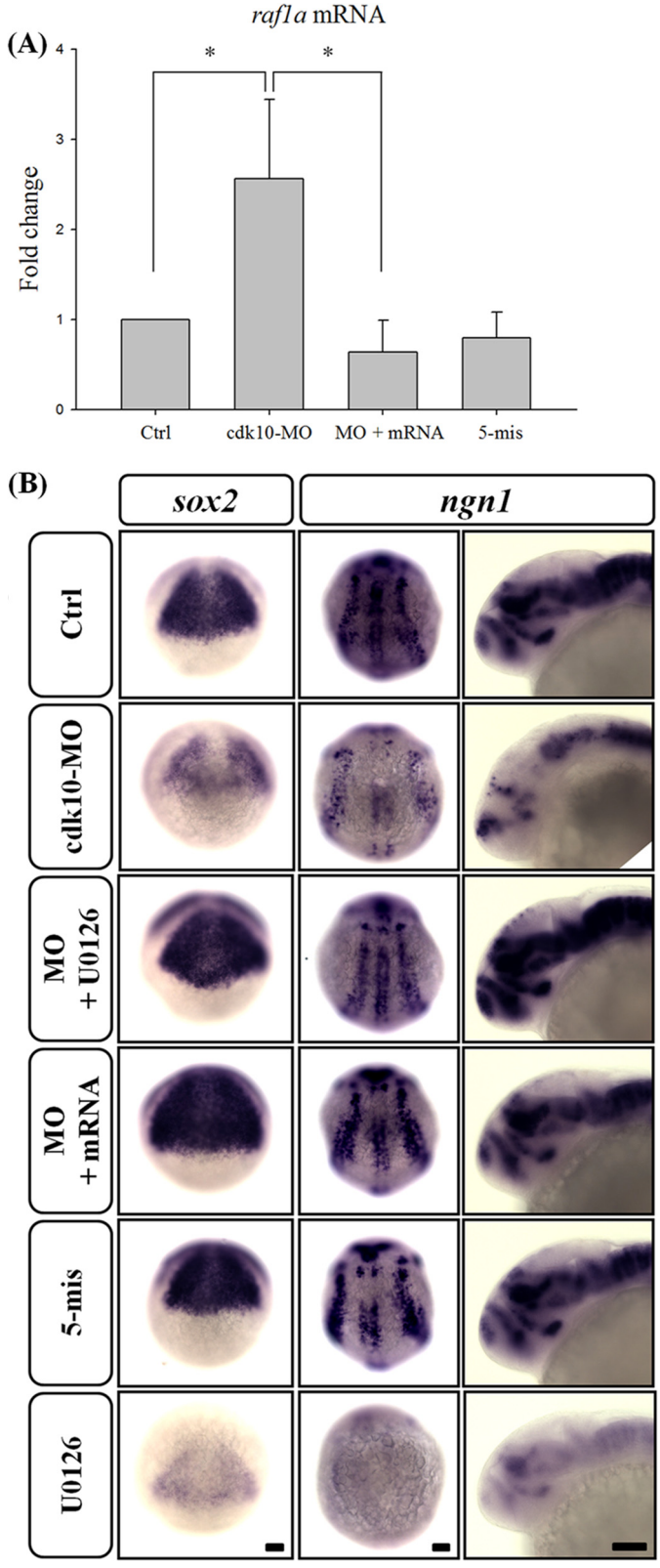


FIGURE 12. **Knockdown of *cdk10* increased the mRNA level of *raf1a*, contributing to the impairment of NPC development.** *A*, quantitative RT-PCR of the *raf1a* gene in control (*Ctrl*), *cdk10* MO, MO + mRNA, and 5-mis MO in embryos at 24 HPF. Expression was normalized to *ef1α*. Knockdown of *cdk10* significantly enhanced the transcription of *raf1a*. *, $p < 0.05$. *Error bars* represent S.D. *B*, embryos received the indicated MO with or without the MEK inhibitor U0126 and were collected at 80% epiboly, tail bud stage, or 24 HPF. Embryos were then subjected to whole-mount *in situ* hybridization using *sox2* or *ngn1* riboprobe. The severe diminution of *sox2* and *ngn1* expression observed in the *cdk10* morphant was effectively eliminated by adding 10 μM U0126. *Scale bars*, 100 μm.

expressed in the brain, eye, and heart (14). The expression of *cdkl1*, which is another cdc2-related protein kinase, is restricted to the floor plate, pronephric ducts, and hypochord at 12 HPF (18). These cdks may exhibit redundant functions during the early developmental stage and perform specific functions during late developmental stages.

Specific morpholinos were designed to knock down *cdk10* to assess the developmental role of *cdk10* in zebrafish. A previous report showed that morpholinos may cause off-target effects that result in neuronal apoptosis because of p53 activation (23). To prevent off-target effects, *p53* MO was co-injected with *cdk10* MO into zebrafish embryos. The knockdown of *cdk10* caused no obvious abnormalities. However, small brains and eyes as well as slight pericardial edema were observed in the morphants. Interestingly, impaired *cdk10* expression resulted in apoptosis in the dorsal regions at the tail bud stage as well as the dorsal neurons and anterior brain regions where *cdk10* is abundantly expressed. These findings were consistent with the whole-mount *in situ* hybridization data. The expression of *sox2*, which is a neural progenitor marker, was found to have expanded and decreased in *cdk10* morphants. Reduced expression of *ngn1*, *emx1*, and *wnt1* in *cdk10* morphants was evident. Expression of a dominant negative form of cyclin H, which decreases cdk7 activities, results in apoptosis in the early stage of zebrafish embryos (14). Previous reports demonstrated the down-regulation of zebrafish *cdk5* by using siRNA, which leads to apoptosis in the brain and a subsequent reduction in the number of primary sensory neurons in the trigeminal ganglion (15, 31). The present study shows that *cdk10* is essential for cell survival in zebrafish.

At 24 HPF, *in situ* hybridization of embryos by using an RNA probe against *islet2* indicated that reduction in the number of RB neurons was more significant in embryos injected with *cdk10* MO than in those injected with control MO. AcTub staining revealed a large reduction in the number of RB neurons along the body axis in *cdk10* morphants. RB neurons are primary sensory neurons that gradually die within the first 2 days of development (32). A previous report showed that *cdk5* may mediate RB neuronal survival. The knockdown of zebrafish *cdk5* by specific siRNAs decreased the number of RB neurons in the spinal cord (15). Phosphorylation of dihydropyrimidinase-like proteins 2 and 3 (DPYSL2 and DPYSL3) by cdk5 and the dual specificity tyrosine-phosphorylated and -regulated kinase (DYRK2) were essential in the proper localization of RB and neural crest cells during neurulation in zebrafish development (33). Conversely, *cdk5* knockdown using specific MOs induced motor neuron development (17). Exposure to perfluorooctane sulfonate resulted in motor neuron defects in zebrafish that may be attributed to increased *cdk5* expression (34). However, no apparent changes in motor neuron expression were observed in *cdk10* morphants. Our findings show that cdk10 exhibits a significant function in the development of the spinal sensory but not the motor nervous system. However, further

investigation must be conducted to determine whether DPYSL2 and DPYSL3 are also downstream targets of cdk10.

Human *CDK10* significantly affects the progression from the G_2/M phase of the cell cycle (8). Overexpression of human CDK10 inhibits the proliferation of human biliary tract cancer (29) and hepatocellular carcinoma (30). By contrast, zebrafish *cdk10* is not involved in G_2/M phase progression. No apparent change in G_2/M phase population was observed in cells overexpressing rodent cdk10 (35). An RNA probe of *cdkn1b*, *cdkn1c*, and *ccnd1* revealed no considerable effects in *cdk10*-deficient embryos. These observations show that *cdk10* exhibits various functional activities in cell cycle progression that differ between human and zebrafish.

RGCs, the output neurons of the retina, contribute to a topographical spatial code that corresponds with their position within the retinal compartments and in higher visual centers of the brain (36). A recent study suggested that elevation of phospho-NR2A(Ser-1232) by Cdk5/p35 contributes to the apoptotic death of RGCs in experimental glaucoma rats (37). In an *in vivo* optic nerve crush model, the combined treatment with the cdk5 inhibitor indolinone A and the Rho-associated protein kinase inhibitor Y-27632 produced a strong stabilizing effect on neuronal survival (38). Administration of the CDK4 inhibitor reduced ischemia-reperfusion injury-induced neural apoptosis (39). By contrast, cdk5 was found to support neuronal survival by Bcl-2 phosphorylation (40) and significantly influence the extension and maintenance of axons as well as the stability and steering of RGCs (41). Administration of the pan-CDK inhibitor AG-012986 also induced apoptosis in retina cells (42).

A previous report showed that depletion of CDK10 expression led to elevated Raf1 levels, consequently activating the MEK-ERK1/2 pathway (12). According to its duration and magnitude, Raf-MEK-ERK1/2 signaling controls diverse cell responses such as proliferation, differentiation, and cell death (29). In the nervous system, the Raf-MEK-ERK1/2 pathway affects the genesis of neural progenitors (29). Treatment with the ERK1/2 inhibitor PD98059 prevented traumatic brain injury in the hippocampus by suppressing ERK1/2 activation (30). Similarly, treatment with U0126 rescued the glutamate-induced neuronal injury by suppressing ERK1/2 activation (43). Death of striatal neurons by dopamine induction has been attributed to ERK1/2 activation (44). Yang *et al.* (45) recently demonstrated that Raf-MEK-ERK1/2 enhancement causes neurogenesis defects in cultured cortical neurons. Similarly, the present study demonstrated that the knockdown of *cdk10* expression triggered *raf1a* expression and decreased expression of neuron progenitor markers such as *sox2* and *ngn1*. Nevertheless, the exposure of embryos to U0126 alone under a nonlethal dosage severely diminished the transcriptional level of *sox2* and *ngn1*, indicating the significance of *raf1a* in maintaining normal neurogenesis. Moreover, Raf1 exhibited a neuroprotective ability during serum withdrawal (46). Inhibition of Raf activities led the otic epithelia to undergo apoptosis, thereby reducing the neurogenesis of the acoustic-vestibular ganglion (47). The results of the present study collectively suggest that fine-tuning the modulation between cdk10 and the Raf-MEK-ERK1/2 axis is necessary for NPC survival.

Thus, the results of ours study emphasize that *cdk10* may positively regulate NPC development. Changes in *cdk10* expression led to NPC apoptosis in the early stages and subsequent impairment in differentiating neurons. Whole-mount *in situ* hybridization revealed a significant reduction in the number of RB neurons in *cdk10* morphants. *Tg* (*huC:gfp*) zebrafish revealed a decrease in the number of RB neurons and RGCs at 24 and 48 HPF. The blocked *cdk10* expression elicited an increase in the *raf1a* level, consequently decreasing NPC survival, whereas the MEK inhibitor reversed these phenomena. In summary, our findings show that *cdk10* significantly affects NPC survival by Raf-MEK-ERK1/2 pathway modulation.

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