Cannabinoid receptor-interacting protein 1 is a regulator of eye and neural development in *Xenopus laevis*

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Abstract

Cannabinoid receptor-interacting protein 1 (CNRIP1, also known as CRIP1) was discovered in human as a novel protein capable of binding to the C-terminal tail of cannabinoid receptor 1 (CNR1, also known as CB1). Although CNRIP1 is evolutionarily conserved throughout vertebrates, very little is known about its physiological function in any organisms. In this study, I have identified a developmental role of CNRIP1 using *Xenopus laevis* embryos. Expression of *Xenopus laevis cnrip1* started at the late blastula stage and was highly restricted to the animal region at the early and late gastrula stages. During neurula stages, *cnrip1* expression was enriched in the neural plate. Knockdown of *cnrip1* in *Xenopus laevis* embryos led to loss of eyes and heads, and shortened anteroposterior axes with curving. At a molecular level, *cnrip1* knockdown strongly suppressed expression of the neural marker *sox2* and the anterior marker *otx2* at the early gastrula stage when neural induction occurs. At the late gastrula stage when eye field induction occurs, expression of eye markers *pax6* and *rax,* as well as that of *sox2* and *otx2,* was suppressed by *cnrip1* knockdown. Overexpression of *cnrip1* was able to expand *sox2*, *otx2*, *pax6* and *rax* expression. Cell lineage specific knockdown revealed that *cnrip1* predominantly functions in cells derived from the dorsal-most animal blastomere to regulate eye development. Moreover, knockdown of *Xenopus laevis cnr1* resulted in eye defects similar to, but milder than, those caused by *cnrip1* knockdown. This study demonstrates that *Xenopus laevis cnrip1* plays a critical role in the early stages of eye and neural development. This study also reveals a functional similarity between CNRIP1 and CNR1 in eye and neural development.

Abbreviations

BMP: bone morphogenetic protein

cnrip1: cannabinoid receptor-interacting protein 1

cnr1: cannabinoid receptor 1

CNS: central nervous system

GFP: green fluorescent protein

GPCRs: G protein-coupled receptors

hCG: human chorionic gonadotropin

MBS: modified Barth's solution

MO: morpholino oligonucleotide

odc: ornithine decarboxylase

otx2: orthodenticle homeobox 2

pax6: paired box 6

PDZ: PSD-95/Disc-large-protein/Zonula occludens-1

qRT-PCR: quantitative reverse transcription polymerase chain reaction

rax: retina and anterior neural fold homeobox

SCG: superior cervical ganglion

sox2: sex determining region Y-box 2

THC: Δ^9-Tetrahydrocannabinol

Wnt8: wingless and int-8

Introduction

Cannabis is the most well-known illicit drug of abuse in the world. The major psychoactive components of cannabis plant, such as Δ^9-Tetrahydrocannabinol (THC), are termed cannabinoids (phytocannabinoids), which are a group of lipophilic compounds (Di Marzo et al., 2004; Velasco et al., 2012). The molecules with cannabinoid-like actions (termed endocannabinoids) also can be found in the nervous and immune systems of the animals and humans (Mechoulam and Parker, 2013). All of the endocannabinoids identified so far are derivatives of long-chain polyunsaturated fatty acids (Di Marzo et al., 2004). They are produced "on demand" from membrane phospholipid precursors (Di Marzo et al., 2004).

The cannabinoids primarily bind to the cannabinoid receptors to exert biological actions. There are two main subtypes, cannabinoid receptor 1 (official symbol CNR1, also commonly known as CB1) and cannabinoid receptor 2 (official symbol CNR2, also commonly known as CB2), both of which belong to the superfamily of G protein-coupled receptors (GPCRs) (Di Marzo et al., 2004; Velasco et al., 2012; Mechoulam and Parker, 2013). In adult rats and humans, CNR1 proteins are widely distributed in the brain, whereas CNR2 proteins are primarily present in the non-neural tissue (Herkenham et al., 1991; Glass et al., 1997; Klein et al., 2003). The endocannabinoid system (which comprises cannabinoid receptors, their endogenous ligands (endocannabinoids), and the machinery that participates in biosynthesis, transportion and inactivation of endocannabinoids) regulates multiple adult physiological processes, including pain-sensation, memory, food intake, mood and reproduction (Di Marzo et al., 2004; Velasco et al., 2012; Mechoulam and Parker, 2013;

Silvestri and Di Marzo, 2013).

It has been reported that the endocannabinoids are present during mammalian embryonic development (Fride, 2004; Harkany et al., 2007). The endocannabinoids are detected in the mouse uterus and play a critical role in embryo implantation (Fride, 2004; Harkany et al., 2007). Administration of synthetic cannabinoids or phytocannabinoids was reported to arrest embryonic growth via CNR1 protein in mice (Fride, 2004; Galve-Roperh et al., 2009). Prenatal exposure to marijuana (produced from cannabis plant) impairs the development of central nervous system (CNS) in the fetuses, suggesting the importance of the endocannabinoid system for neural development (Harkany et al., 2007). However, little is known about the mechanism by which the endocannabinoid system regulates embryonic development.

The expression of *Cnr1* in the embryos has been investigated more thoroughly than that of *Cnr2* (Fride, 2004). C*nr1* is expressed in mammalian developing brain (Fride, 2004). In chick embryos, *CNR1* transcript is present in the anterior neural plate during gastrulation and in the territories of presumptive forebrain, midbrain, and hindbrain during neurulation (Psychoyos et al., 2012). In *Xenopus* embryos, *cnr1* expression continues to be relatively low until the early tailbud stage but is significantly increased from the late tailbud stage (Migliarini et al., 2006). Although *Cnr1*-deficient mice are apparently normal, they have defects of corticothalamic and thalamocortical projections and adult neurogenesis (Jin et al., 2004; Wu et al., 2010), and show abnormal memory-related behaviors (Marsicano et al., 2002). However, the role of *Cnr1* in embryonic development is obscure.

Cannabinoid receptor-interacting protein 1 (official symbol CNRIP1, also known as CRIP1) is a novel binding partner of CNR1 (Niehaus et al., 2007). It specifically

interacts with the C-terminus of CNR1 but not with CNR2. Human *CNRIP1* gene is found on chromosome 2. Alternative splicing produces two different transcripts, *CNRIP1a* (containing exons 1, 2 and 3a) and *CNRIP1b* (containing exons 1, 2 and 3b), which encode 164 and 128 amino acids respectively. *CNRIP1a* exists throughout the vertebrates, whereas so far *CNRIP1b* has been discovered only in primates (Niehaus et al., 2007). *Xenopus laevis cnrip1* corresponds to human *CNRIP1a. Xenopus laevis* Cnrip1 protein contains 162 amino acids. It shows 67% amino acid identity with human CNRIP1a protein (Figure 1). It also shows 82%, 70%, 67% and 67% amino acid identity with its orthologs in chick, zebrafish, mouse and rat, respectively (Figure 1). The C-terminal tail of human CNRIP1a but not CNRIP1b protein contains a predicted PSD-95/Disc-large-protein/Zonula occludens-1 (PDZ) class I ligand (Niehaus et al., 2007). However, the public database search reveals that the PDZ class I ligand cannot be found in *Xenopus laevis* Cnrip1, chick CNRIP1 and zebrafish Cnrip1 (Figure 1A), suggesting that the potential for interactions with PDZ domains is not evolutionarily conserved.

The expression pattern of CNRIP1 has not been fully studied. Previous reports showed that in adult mice, CNRIP1a protein is highly expressed in the brain (Niehaus et al., 2007) and it is also expressed in the retina (Hu et al., 2010). CNRIP1 protein is also detectable in the developing brain of fetal mice (Keimpema et al., 2010). In cultured superior cervical ganglion (SCG) neurons that are isolated from rat, exogenously expressed human CNRIP1a proteins were found to be enriched near the plasma membrane and colocalize with exogenous CNR1 protein (Niehaus et al., 2007). This colocalization cannot be seen in the mouse retina, but the endogenous CNRIP1 proteins are located in close proximity to endogenous CNR1 protein in the pre-synaptic

terminals of the retina cone photoreceptors (Hu et al., 2010).

The functional interaction between CNRIP1 and CNR1 remains vague. It has been shown that exogenously expressed human CNRIP1 can attenuate constitutive CNR1-mediated inhibition of calcium channels in the cultured rat SCG neurons, suggesting that CNRIP1 may modulate constitutive CNR1 activity in the CNS (Niehaus et al., 2007). Another report shows that knockdown of *Cnr1* led to upregulation of *Cnrip1* expression in rat medium spiny neurons, which might suggest a role for modulation of CNRIP1 function by CNR1 (Blume et al., 2013).

In the preliminary experiment done by my lab mates (unpublished data), ectopic expression of two well-known secreted developmental regulators Chordin (an antagonist of BMP) and Wnt8 was performed in *Xenopus laevis* animal cap explants (which contains presumptive neuroectoderm) to screen for their target genes. And then *cnrip1* was found to be a potential target gene of Chordin and Wnt8. Therefore the aim of this study is to identify the developmental role of *cnrip1* by using *Xenopus laevis* embryos.

A

B

Protein sequence identity of CNRIP1 between Xenopus laevis and other species

Description	Identity (%)
CNRIP1 [Gallus gallus]	82%
CNRIP1 [Danio rerio]	70%
CNRIP1 [Mus musculus]	67%
CNRIP1 a [Homo sapiens]	67%
CNRIP1 [Rattus norvegicus]	67%

Figure 1. Multiple protein sequence alignment of CNRIP1 from different species.

(A) The protein sequence alignment was obtained using T-Coffee and shaded with Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The conserved residues are marked with black shades. The similar residues are marked with grey shades. (B) Percentages of identity of CNRIP1 between *Xenopus laevis* and other species were calculated based on protein sequence alignment.

Materials and methods

Collection and fertilization of eggs

The female frogs were induced to spawn by injection of human chorionic gonadotropin (hCG) (Mochida company). The eggs were collected into a Petri dish. The freshly obtained eggs were mixed with a fragment of testis for *in vitro* fertilization. The fertilized embryos were cultured in 0.1 × Modified Barth' Solution (MBS) and staged according to the method described by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Plasmids and constructs

The full length coding sequences of *Xenopus laevis cnrip1* (GenBank accession number BC082678) and *cnr1* (GenBank accession number AY098532) were obtained by PCR from stage 33/34 cDNA, and then cloned into the EcoRI-XhoI sites of the pCS4 vector, in which the BamHI site of the pCS2+ vector (http://sitemaker.umich.edu/dlturner.vectors) was replaced by a BglII site.

The sequences of primers for *cnrip1* cloning were as follows:

forward, 5'-ATACCGGAATTCATGGGGGAGATCCCGAGCCTGGTCA-3';

reverse, 5'-ATACCGCTCGAGTTAATGGAATATTTCTTTGTTTATC-3'.

The sequences of primers for *cnr1* cloning were as follows:

forward, 5'-ATACCGGAATTCATGAAGTCAATTCTGGATGGC-3';

reverse, 5'-ATACCGCTCGAGTTATACTGCTTCTGCAGATGTGTC-3'.

The sequences of primers for generating the N-terminally *myc*-tagged *cnrip1* construct

were as follows:

forward, 5'-GGAAGATCTATGGGGGAGATCCCGAGCCT-3';

reverse, 5'-GGTAGATCTTTAATGGAATATTTCTTTGTTTATC-3'.

The sequences of primers for generating the C-terminally *myc*-tagged *cnrip1* construct were as follows:

forward, 5'-GGTAGATCTGGCCGCAAGAAATGGGGGAGATCCCGA-3';

reverse, 5'-GGTAGATCTATGGAATATTTCTTTGTTTATCTCCA-3'.

The underlines indicate the sequences complementary to *cnrip1* or *cnr1* genes. The PCR products for *myc*-tagged constructs were digested by BglII and inserted into the pCS4 vector containing the myc encoding sequence upstream or downstream of the BglII site. The coding regions of *Xenopus laevis sox2*, *otx2*, *pax6* and *rax* (their GenBank accession numbers are AF005476, BC077357, BC041712 and AF001048, respectively) were amplified by PCR by using stage 33/34 cDNA and inserted in a reverse orientation into pCS4 vectors for the antisense probe synthesis from the SP6 promoter.

Preparation of synthetic RNAs and morpholino oligos for microinjection

For mRNA injection, all plasmids (pCS4-*cnrip1*, pCS4-*myc-cnrip1*, pCS4-*cnrip1-myc*) were linearized and transcribed with SP6 polymerase, using mMESSAGE mMACHINE *in vitro* transcription kits (Ambion). All morpholino oligonucleotides (MOs) were designed and produced by Gene Tools. The sequences of the MOs were designed as follows:

cnrip1 MO, 5'-GATCTCCCCCATTCTTGCGGCC-3';

cnrip1-5mis MO, 5'-GATCTaCCaCATTTaTTGaGaCC-3';

cnr1 MO1, 5'-GGCCATCCAGAATTGACTTCATTAC-3';

cnr1 MO2, 5’-CAGGTGGAATCAAATTCTCTTTGGA-3’;

cnr1-5mis MO, 5’-GGCaATaCAcAATTcACTTaATTAC-3’;

control MO, 5’-CCTCTTACCTCAGTTACAATTTATA-3’.

The underlines indicate the sequences complementary to the predicted start codon. Lower case letters indicate mismatched bases.

Quantitative RT-PCR

Total RNA was isolated from embryos by extraction with TRIzol reagent (Invitrogen), and cDNA was synthesized by using reverse transcriptase M-MLV (Invitrogen) with random primers. Quantitative RT-PCR (qRT-PCR) was performed with QuantiTect SYBR Green PCR Kit (Qiagen). The gene expression levels were normalized to those of *ornithine decarboxylase* (*odc*). The statistical differences were analyzed by the unpaired Welch t-test using the EXCEL Software (Microsoft). The sequences of primers used for qRT-PCR were as follows:

cnrip1, forward 5’-TCGGGAGCTTCGAGACTGTATGGC-3’,

reverse 5’-AGGCTGCGGGTTTCGTTGGGTTTG-3’;

sox2, forward 5’-TACATGAACGGCTCGCCTACC-3’,

reverse 5’-CGCGAATGGGAAGAAGAGGTG;

otx2, forward 5’-TCTCAAGCAACCGCCATACGC-3’,

reverse 5’-CTTTCCCTCCTCTGTTTCCTGGG-3’;

pax6, forward 5’-CGGCAGCGACTTAGACATGC-3’,

reverse 5’-GCGCCAGTTCCACGATCTTC-3’;

rax, forward 5’-GACGACGAACAACAGCCCAAG-3’,

reverse 5’-TTCTCGAATGCCCTCTCCAGC-3’;

cnr1, forward 5'-TGGGCACATTCACAGTCTTGG-3',

reverse 5'-TGCCAATGAAGTGGTAGGAGGG-3';

odc (GenBank accession number BC044004), forward

5'-TGCAAGTTGGAGACTGGATG-3',

reverse 5'-CATCAGTTGCCAGTGTGGTC-3'.

Immunoblotting

Embryos were extracted with lysis buffer (20 mM Tris-HCl pH 7.5, 27 mM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EGTA, 6.4 mM β-glycerophosphate, 2.7 mM $Na_4P_2O_7$, 1 mM DTT, 10 mM NaF, 1 mM vanadate, 1% NP-40) containing a cocktail of protease inhibitors (Sigma). Samples were loaded and run on 12% SDS-polyacrylamide gels, transferred to PVDF membrane (Millipore). The primary antibodies used were mouse anti-Myc (1:2000; Santa Cruz Biotechnology, 9E10) and mouse anti-GFP (1:2000; Clontech, JL-8). Sheep anti-mouse IgG HRP-conjugated (1:10,000; GE Healthcare) was used as the secondary antibody. The images were obtained by LAS 4000.

Whole-mount *in situ* hybridization

The embryos were fixed in MEMFA solution for 1.5-2 hours, dehydrated and stored in methanol. The antisense DIG-labeled RNA probes for *cnrip1* and *cnr1* were transcribed *in vitro* by using T7 RNA polymerase (Takara). The antisense DIG-labeled RNA probes for *sox2*, *otx2*, *pax6* and *rax* were transcribed by SP6 RNA polymerase (Takara). Whole-mount *in situ* hybridization was carried out according to the standard protocol (Sive, 2000). Colour reactions were performed and the embryos were bleached before scoring.

Results

The expression pattern of *cnrip1* in *Xenopus laevis* embryos

The expression pattern of *Xenopus laevis cnrip1* during embryonic development was examined. The temporal expression of *cnrip1* was analyzed by quantitative RT-PCR (qRT-PCR) with cDNA from stage 2 to stage 33/34 (Figure 2). At stage 2 (the 2-cell stage), *cnrip1* expression was barely detectable. At stage 9 (the late blastula stage, immediately after the initiation of zygotic transcription), *cnrip1* expression stayed at a low level. At stage 10.5 (the early gastrula stage), the expression level of *cnrip1* increased sharply. The expression of *cnrip1* was kept at a high level until stage 12 (the late gastrula stage). At stage 15 (the mid-neurula stage), *cnrip1* expression level greatly dropped down, but afterwards it increased gradually again and reached a maximum at stage 33/34 (the late tailbud stage).

The spatial expression of *cnrip1* was analyzed by whole-mount *in situ* hybridization. At stages 10.5 and 12, *cnrip1* was distributed in the animal region (the presumptive ectoderm) (Figure 3A, B, D, E). At stage 15, *cnrip1* expression was observed in the neural plate, with higher level in the anterior part than in the posterior part (Figure 3G, H). At stage 19 (the late neurula stage), *cnrip1* mRNA was enriched in the whole neural plate, and its level was still kept higher in the anterior part than in the posterior part (Figure 3J, K). At stage 25 (the early tailbud stage), the expression of *cnrip1* was observed in the neural tube (higher in the rostral than in the caudal), notochord (higher in the rostral than in the caudal), trigeminal placode and olfactory placode (Figure 3M-P). At stage 33/34, *cnrip1* was expressed in the forebrain, pineal gland, midbrain,

hindbrain, eyes, trunk neural tube (spinal cord), the posterior end of the notochord, olfactory placode and the trigeminal placode (Figure 3R-T, V). Transverse trunk section of the middle part of a stage 25-embryo showed that *cnrip1* mRNA was abundant in the neural tube and notochord (Figure 3O). The trunk section also indicated that in the neural tube, *cnrip1* was expressed more strongly in the alar plate and the basal plate than in the roof plate and the floor plate. Similar distribution was observed in stage 33/34-embryos, except that *cnrip1* expression was downregulated in the notochord (Figure 3T). The sense probe of *cnrip1* used as a negative control at each stage showed no signal (Figure 1C, F, I, L, Q, U).

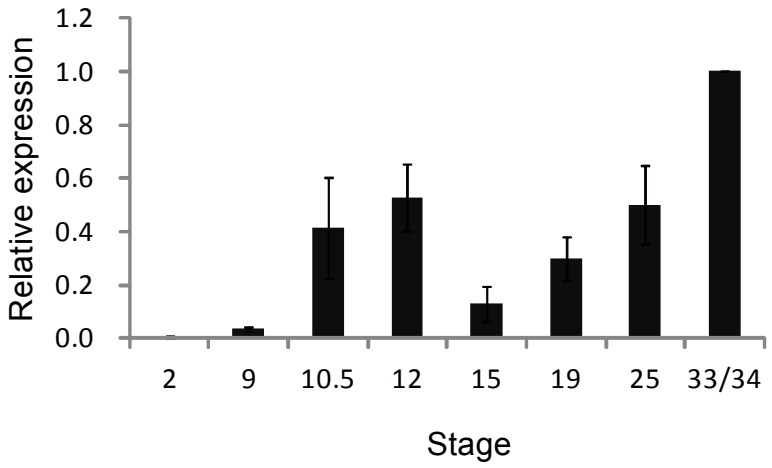

Figure 2. Temporal expression of *cnrip1* in early *Xenopus laevis* development.

cnrip1 expression was analyzed by qRT-PCR. Samples were normalized to *ornithine decarboxylase* (*odc*) and values were shown as a ratio relative to the normalized expression level at stage 33/34 (relative expression). The average ratio of each stage from three independent experiments is shown. The error bar indicates SD.

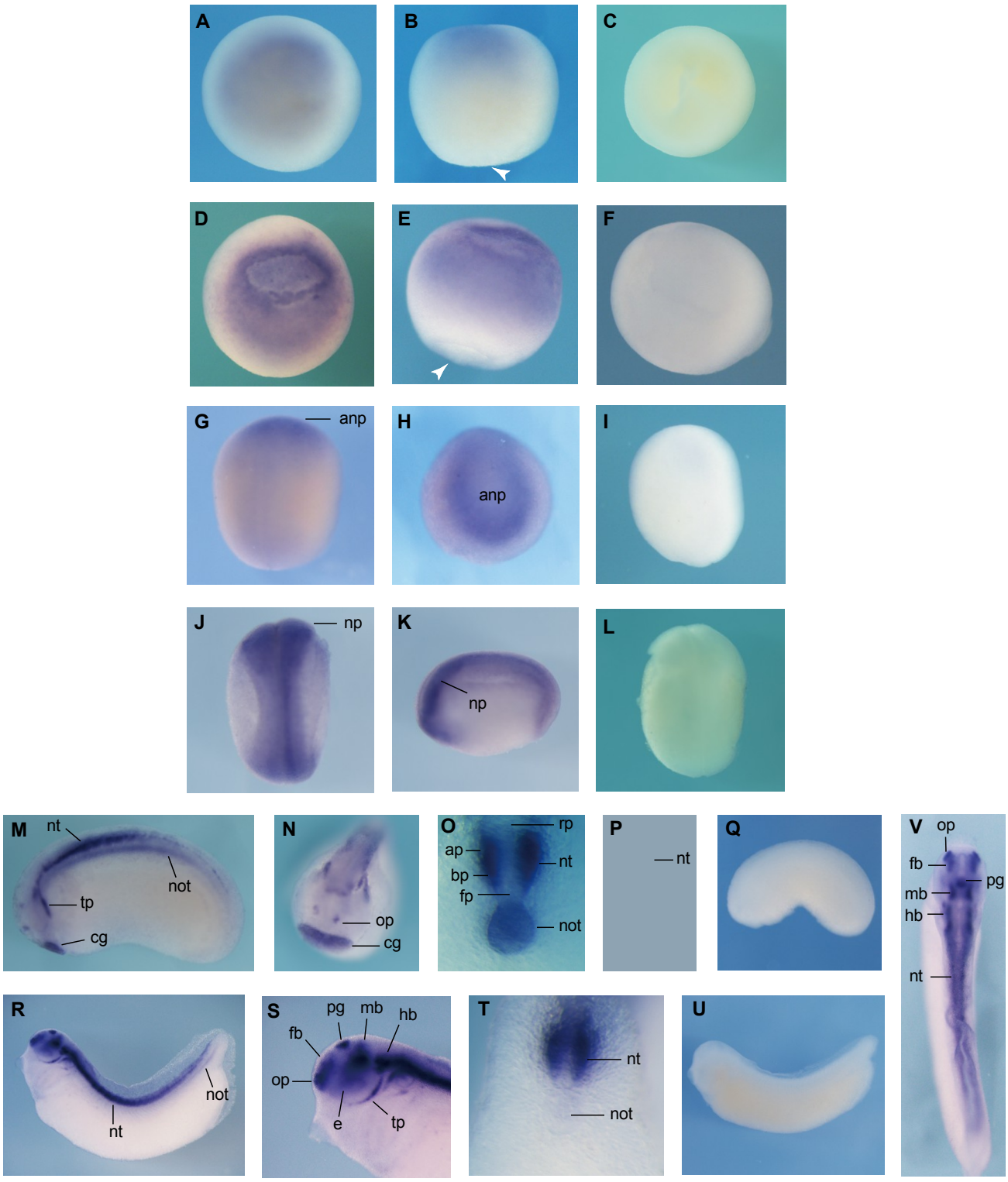

Figure 3. Spatial expression of *cnrip1* in early *Xenopus laevis* development.

Spatial expression was analyzed by whole-mount *in situ* hybridization. Antisense RNA probe of

cnrip1 was used at the stages 10.5 (A,B), 12 (D,E), 15 (G,H), 19 (J,K), 25 (M-P), 33/34 (R-T,V). (A,D) Animal views. (B,E) Lateral views with blastopore downwards (arrowhead). (G,J,P,V) Dorsal views. (H,N) Anterior views. (K,M,R) Lateral views with dorsal upwards. (O) A trunk section of a stage-25 embryo. (S) Magnified view of the stage-33/34 embryo shown in R. (T) A trunk section of a stage-33/34 embryo. (C,F,I,L,Q,U) Sense RNA probe of *cnrip1* was used and showed no signal at every stage. (C) Stage 10.5, animal view. (F) Stage 12, animal view. (I) Stage 15, dorsal view. (L) Stage 19, dorsal view. (Q) Stage 25, lateral view. (U) Stage 33/34, lateral view. anp, anterior neural plate; np, neural plate; nt, neural tube; not, notochord; cg, cement gland; tp, trigeminal placode; op, olfactory placode; ap, alar plate; bp, basal plate; fp, floor plate; rp, roof plate; fb, forebrain; pg, pineal gland; mb, midbrain; hb, hindbrain; e, eye.

Knockdown of *cnrip1* impairs eye and neural development

In order to examine the role of *Xenopus laevis cnrip1* during early embryogenesis, loss-of-function study of *cnrip1* was performed by injecting the antisense morpholino oligo against *cnrip1* (cnrip1 MO), which was designed to bind with the ATG site of *cnrip1* mRNA and thus inhibit the translation of the Cnrip1 protein. Firstly, the efficacy of cnrip1 MO was examined. At 2-cell stage, control MO or cnrip1 MO (40 ng each/embryo) was injected with *cnrip1-myc* mRNA, which contains the cnrip1 MO-targeted 5'UTR sequence, the coding sequence of *cnrip1* and the sequence encoding a *myc* tag at the C-terminus. *GFP* mRNA was also co-injected to serve as an injection control. Immunoblotting analysis showed that cnrip1 MO significantly inhibited the translation of *cnrip1-myc* mRNA (Figure 4A, lanes 4 and 5), indicating that cnrip1 MO is efficient. Because *cnrip1* was mainly expressed in the nervous system (Figure 3), 10 ng of control MO, cnrip1 MO, or cnrip1-5mis MO (a five-base mismatch negative control which was used to examine the specificity of cnrip1 MO) was injected into the animal region of the two dorsal blastomeres of 4-cell stage embryos, which forms most of neural structures at later stages. At stage 19, the cnrip1 MO-injected embryos displayed defects in neural tube closure, whereas control MO- and cnrip1-5mis MO-injected embryos developed normally (Figure 4B). At stage 37/38, cnrip1 MO-injected embryos, but not control MO- or cnrip1-5mis MO-injected embryos, exhibited remarkable developmental abnormalities, including the eye malformation (strongly reduced or completely absent), head defects, and shortened and curved anteroposterior axes, all of which were simultaneously observed in almost all embryos (Figure 4C).

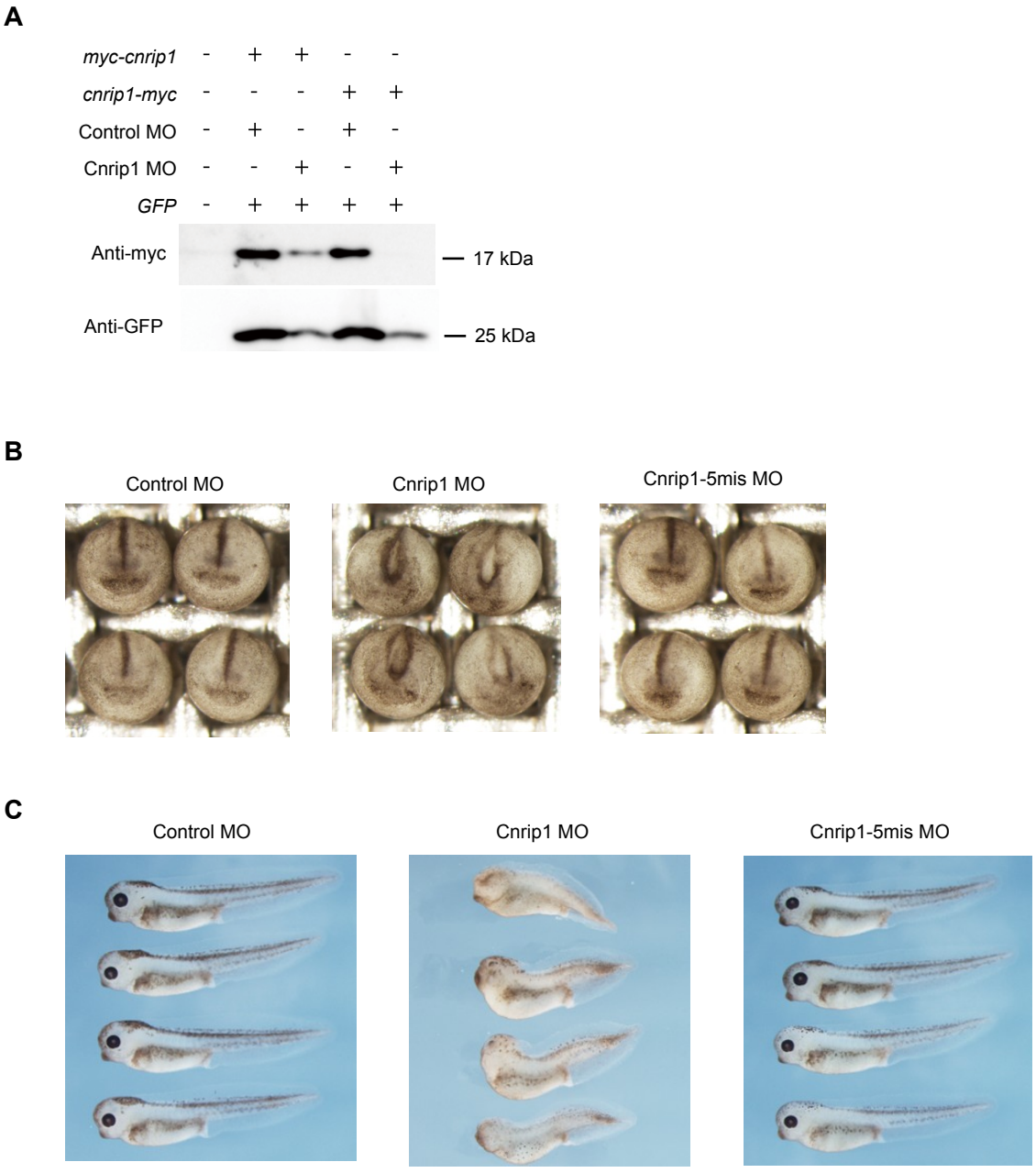

Figure 4. *cnrip1* knockdown disrupts eye and neural development.

(A) Control MO or cnrip1 MO (40 ng each/embryo) was co-injected with N- or C-terminally *myc*-tagged *cnrip1* mRNA (1 ng each/embryo) into the animal regions of the 2-cell embryos. *GFP* mRNA (200 pg/embryo) was also co-injected to check whether the injection was successful and whether the protein loading was controlled. The embryos were collected at stage

10.5 and analyzed by western blot. (B-C) Control MO, cnrip1 MO or cnrip1-5mis MO was injected into the animal regions of the two dorsal blastomeres of 4-cell embryos (10 ng each/blastomere). The representative results from three independent experiments are shown. (B) Stage 19, anterior views (dorsal upwards). The embryos injected with control MO developed normally (100%, n=65). The embryos injected with cnrip1 MO displayed obviously open neural tube (96%, n=72). The embryos injected with cnrip1-5mis MO developed normally (100%, n=70). (C) Stage 37/38, lateral views (anterior to left). The embryos injected with control MO developed normally (100%, n=65). The embryos injected with cnrip1 MO displayed head defects, eye defects, and shortened curved body axes (100%, n=72). The embryos injected with cnrip1-5mis MO developed normally (100%, n=70).

cnrip1 knockdown reduces the expression of *sox2*, *otx2*, *pax6* and *rax*

To investigate the underlying molecular mechanism of *cnrip1* function in the early embryonic development, I examined whether *cnrip1* knockdown affected the expression of a set of key developmental genes, including *sox2* (the pan-neural plate marker), *otx2* (the anterior neural marker), *pax6* (the eye and neural marker) and *rax* (also known as *rx1*, the eye marker).

Sox2 (Sex determining region Y-box 2) protein contains a single HMG (high mobility group) domain that binds to specific DNA sequence (Kamachi et al., 2000). It starts to be expressed in the dorsal ectoderm (neuroectoderm) at stage 10.5 (when neural induction occurs) and is pan-neurally expressed throughout embryonic stages in *Xenopus laevis* embryos (Mizuseki et al., 1998; Kishi et al., 2000). It plays a critical role in neural induction and differentiation (Mizuseki et al., 1998; Kishi et al., 2000). As shown in Figure 5A, qRT-PCR results showed that an obvious reduction of *sox2* expression was caused by cnrip1 MO at stages 10.5 and 12.5. To examine the spatial change of *sox2* expression, control MO or cnrip1 MO was injected into one left dorsal blastomere of the 4-cell embryos, and then injected embryos were analyzed by whole-mount *in situ* hybridization. Results showed that at stages 10.5 and 12.5, in the cnrip1 MO-injected embryos, *sox2* expression was greatly reduced in the injected sides, compared with that in the uninjected sides, whereas *sox2* expression was normal in the control MO-injected embryos (Figure 5A).

otx2 (*orthodenticle homeobox 2*) encodes a homeodomain transcription factor that specifies anterior body region (Pannese et al., 1995). It starts to be expressed in the presumptive anterior neuroectoderm at stage 10.5 in *Xenopus* embryos (Pannese et al., 1995; Zuber et al., 2003). Both qRT-PCR and whole-mount *in situ* hybridization

showed that cnrip1 MO mildly but significantly reduced *otx2* expression at stages 10.5 and 12.5 (Figure 5B).

Pax6 (Paired box 6), the paired homeodomain transcription factor, and Rax (Retina and anterior neural fold homeobox), the homeodomain transcription factor, are important regulators of eye development (Chow et al., 1999; Bailey et al., 2004). Pax6 and Rax, together with other eye field transcription factors, specify the eye field in the anterior neural plate at stage 12.5 in *Xenopus laevis* embryos (Zuber et al., 2003). *pax6* expression is observed in the anterior neuroectoderm and in the two medial stripes in the presumptive neural tube at late gastrula to neurula stages, and then it is distributed in the eye, brain and spinal cord at later stages (Hirsch and Harris, 1997; Zuber et al., 2003). *rax* is expressed in the eye field domain at late gastrula to neurula stages, and then expressed in the developing retina, pineal gland and ventral hypothalamus at later stages (Bailey et al., 2004). In order to determine how *cnrip1* affected eye development, expression changes of *pax6* and *rax* were examined. The results of qRT-PCR and whole-mount *in situ* hybridization showed that *cnrip1* knockdown reduced the expression of *pax6* and *rax* at stages 12.5 and 24 (Figure 5C, D). Significantly, the reductions in the expression of *pax6* and *rax* at stage 12.5 were remarkable (Figure 5C, D), suggesting that *cnrip1* is critical for the initial phase of eye and neural development.

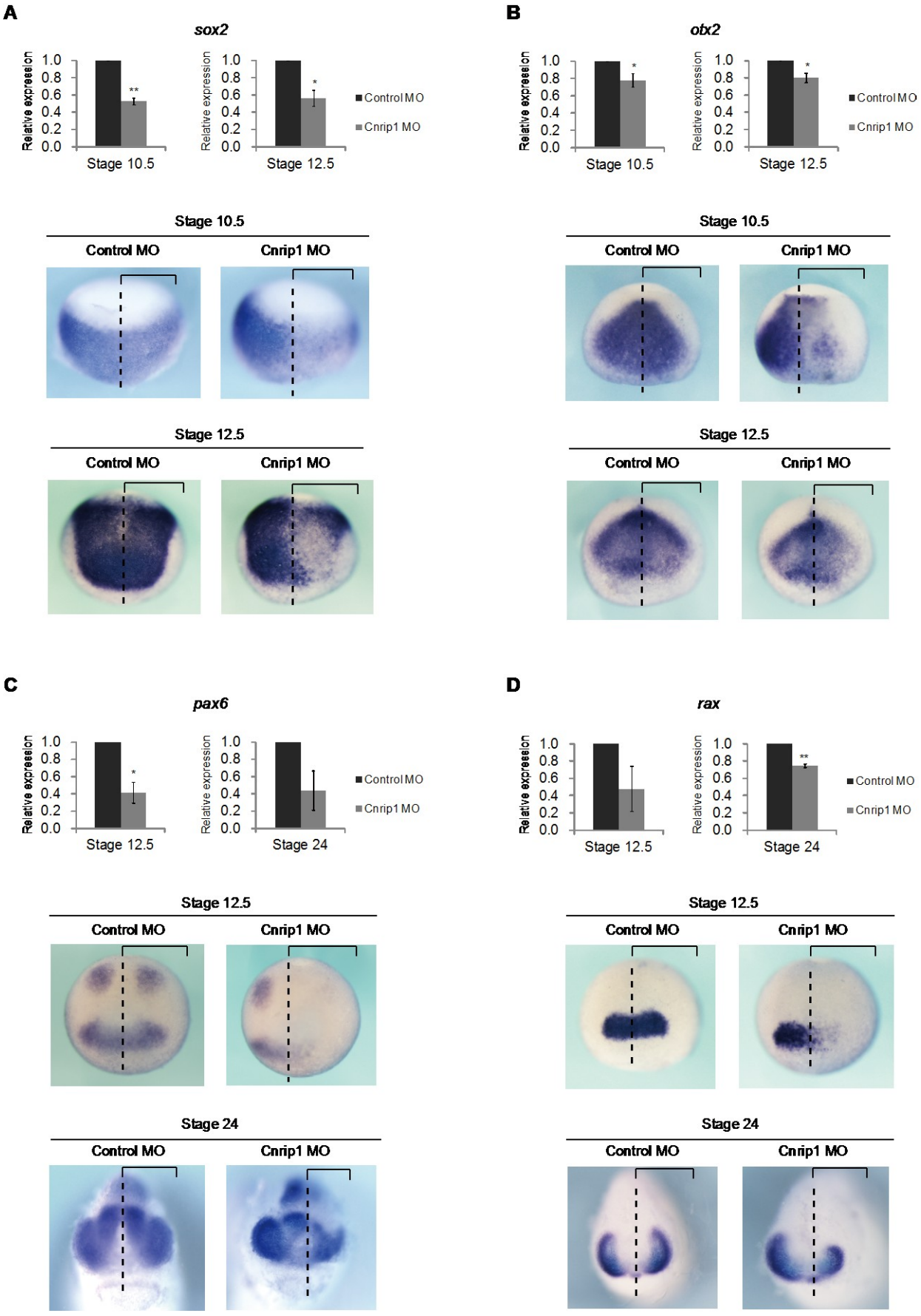

Figure 5. *cnrip1* knockdown reduces the expression of *sox2*, *otx2*, *pax6* and *rax*.

(A-D) The expression of *sox2*, *otx2*, *pax6* and *rax* was analyzed by qRT-PCR and whole-mount

in situ hybridization. (A) *sox2* (stage 10.5 and 12.5). (B) *otx2* (stage 10.5 and 12.5). (C) *pax6* (stage 12.5 and 24). (D) *rax* (stage 12.5 and 24). For qRT-PCR analysis, control MO or cnrip1 MO was injected into the animal region of two dorsal blastomeres at 4-cell stage (10 ng each/blastomere). The expression level of each gene was normalized to that of *odc*. Values are mean ± SD of three independent experiments; **, $p<0.01$; *, $p<0.05$. For whole-mount *in situ* hybridization analysis, control MO or cnrip1 MO was injected into the animal region of the left dorsal blastomeres at the 4-cell stage (10 ng each). The representative results from two (*sox2*, stage 10.5, n=20; *otx2*, stage 10.5, n=20) or three (*sox2*, stage 12.5, n=24; *otx2*, stage 12.5, n=24; *pax6*, stage 12.5, n=30; *pax6*, stage 24, n=24; *rax*, stage 12.5, n=24; *rax*, stage 24, n=24) independent experiments are shown. The expression of *sox2*, *otx2*, *pax6* and *rax* was reduced in the injected sides of all cnrip1 MO-injected embryos. Anterior views (dorsal upwards). MO-injected sides are indicated by the black brackets. The midlines of the embryos are indicated by the dashed lines.

Overexpression of *cnrip1* causes expansion of *sox2*, *otx2*, *pax6* and *rax* expression

To examine the effects of *cnrip1* overexpression on the expression of marker genes, *cnrip1* mRNA was injected into the left dorsoanimal blastomere of the 8-cell embryos and then whole-mount *in situ* hybridization was performed to analyze the expression of *sox2*, *otx2*, *pax6* and *rax*. The uninjected right sides were used as controls.

Results showed that at stage 12.5, the expression domain of *sox2* was laterally expanded in the injected side in 29% of injected embryos (Table 1 and Figure 6A, second and third left). No obvious change was observed in the rest of injected embryos (Table 1 and Figure 6A, right three panels). This result suggests a potential of positive effect of *cnrip1* on *sox2* expression.

At stage 12.5, an expansion of *otx2* was observed in 15% of injected embryos (Table 1 and Figure 6B, second and third left). At stage 24, the percentage of embryos with expanded domain of *otx2* was increased to 23% (Table 1 and Figure 6C, second and third left). Notably, the *otx2* expression domains in the brain and the eye became difficult to distinguish from each other in the injected side at this stage (Figure 6C, second and third left). The result also showed that at stage 24, in 14% of injected embryos, *otx2* expression domain in the brain was expanded in the brain, but simultaneously it was reduced in the eye (Table 1 and Figure 6C, fourth left). Although the eye field is derived from the forebrain domain (Zuber et al., 2003), the expansion of *otx2* expression in the brain was not always accompanied by the expansion in the eye. This may be simply due to the different distribution efficacies of the injected *cnrip1* mRNA in the different regions of embryos. Another possibility may be that the effect of *cnrip1* on *otx2* expression is region-dependent.

At stage 24, in 31% of injected embryos, *pax6* expression in the injected side was expanded (Table 1 and Figure 6D, second and third left). In these embryos, *pax6* expression domains in the brain, the eye and the trunk neural tube became less distinguishable from each other, like the *otx2* expression domains in the injected side (compare with Figure 6C, second and third left). The result also showed that in 34% of injected embryos, *pax6* expression domain in the injected sides was expanded in the brain, but it was reduced in the eye (Table 1 and Figure 6D, fourth left).

At stage 24, the percentage of the embryos with expanded *rax* expression in the injected side was 47% (Table 1 and Figure 6E, second and third left). The reduction of *rax* expression was observed in 10% of injected embryos (Table 1 and Figure 6E, fourth left).

These results suggest that *cnrip1* has a tendency to induce expanded expression of *sox2*, *otx2*, *pax6* and *rax* in early eye and neural development.

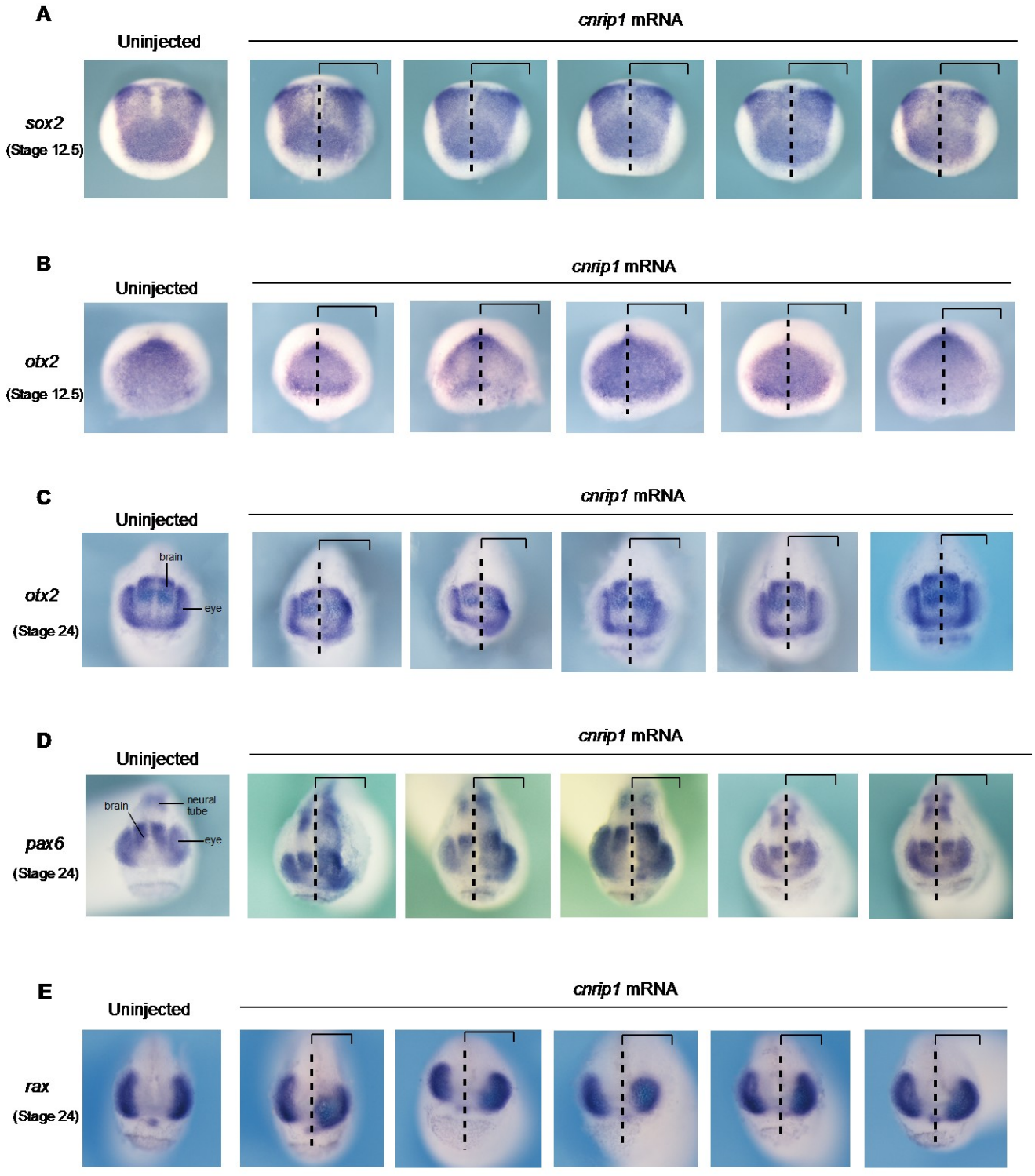

Figure 6. Effects of *cnrip1* overexpression on the expression of *sox2*, *otx2*, *pax6* and *rax*.

Whole-mount *in situ* hybridization analysis was performed on embryos uninjected or injected with 500 pg of *cnrip1* mRNA into the left dorsoanimal blastomere at the 8-cell stage. The

representative results from two (B,C) or three (A,D,E) independent experiments are shown. Anterior views with dorsal upwards. The black brackets indicate mRNA-injected left sides. The dashed lines indicate midlines of embryos. (A) *sox2* expression at stage 12.5. Injected sides of embryos displayed expanded expression (second and third left) or no obvious change (fourth left to rightmost). (B) *otx2* expression at stage 12.5. Injected sides of embryos displayed expanded expression (second and third left) or no obvious change (fourth left to rightmost). (C) *otx2* expression at stage 24. Injected sides of embryos displayed expanded expression (second and third left), expanded expression in the brain but reduced expression in the eye (fourth left), or no obvious change (rightmost and second right). (D) *pax6* expression at stage 24. Injected sides of embryos displayed expanded expression (second and third left), expanded expression in the brain but reduced expression in the eye (fourth left), or no obvious change (rightmost and second right). (E) *rax* expression at stage 24. Injected sides of embryos displayed expanded expression (second and third left), reduced expression (fourth left), or no obvious change (rightmost and second right). The above results were summarized in Table 1.

Table 1. Summaries of the results shown in Figure 6A-E.

Gene name	Stage of embryos	Total number of embryos (n=)	Embryos with expanded expression (%)	Embyros with expanded expression in brain but reduced expression in eye (%)	Embryos with no obvious change (%)
sox2	st 12.5	24	29% (7/24)		71% (17/24)
otx2	st 12.5	20	15% (3/20)		85% (17/20)
otx2	st 24	22	23% (5/22)	14% (3/22)	64% (14/22)
pax6	st 24	32	31% (10/32)	34% (11/32)	34% (11/32)
rax	st 24	30	47% (14/30)	10% (3/30)	43% (13/30)

Eye defects in cnrip1 MO-injected embryos can be partly alleviated by co-injection of *cnrip1* mRNA

To test whether the eye phenotypes caused by cnrip1 MO can be rescued by exogenously expressed *cnrip1*, we generated an N-terminally *myc*-tagged *cnrip1* construct (*myc-cnrip1*), which contains the full coding sequence of *cnrip1* but lacks the 5'UTR sequence targeted by cnrip1 MO. Cnrip1 MO did not fully deplete Myc-Cnrip1 protein (see Figure 4A, lanes 2 and 3), indicating that *myc-cnrip1* is relatively resistant to cnrip1 MO. Next, cnrip1 MO was injected with or without *myc-cnrip1* mRNA into the left dorsoanimal blastomere at the 8-cell stage. Cnrip1 MO alone caused severe eye defects (absent eyes, very small eyes) in 64% of injected embryos (Figure 7A, B). Co-injection of cnrip1 MO and *myc-cnrip1* mRNA decreased the percentage of embryos with severe eye defects to 51% (Figure 7A, B). Because the rescue was statistically significant although partial, the phenotypes caused by cnrip1 MO are specific.

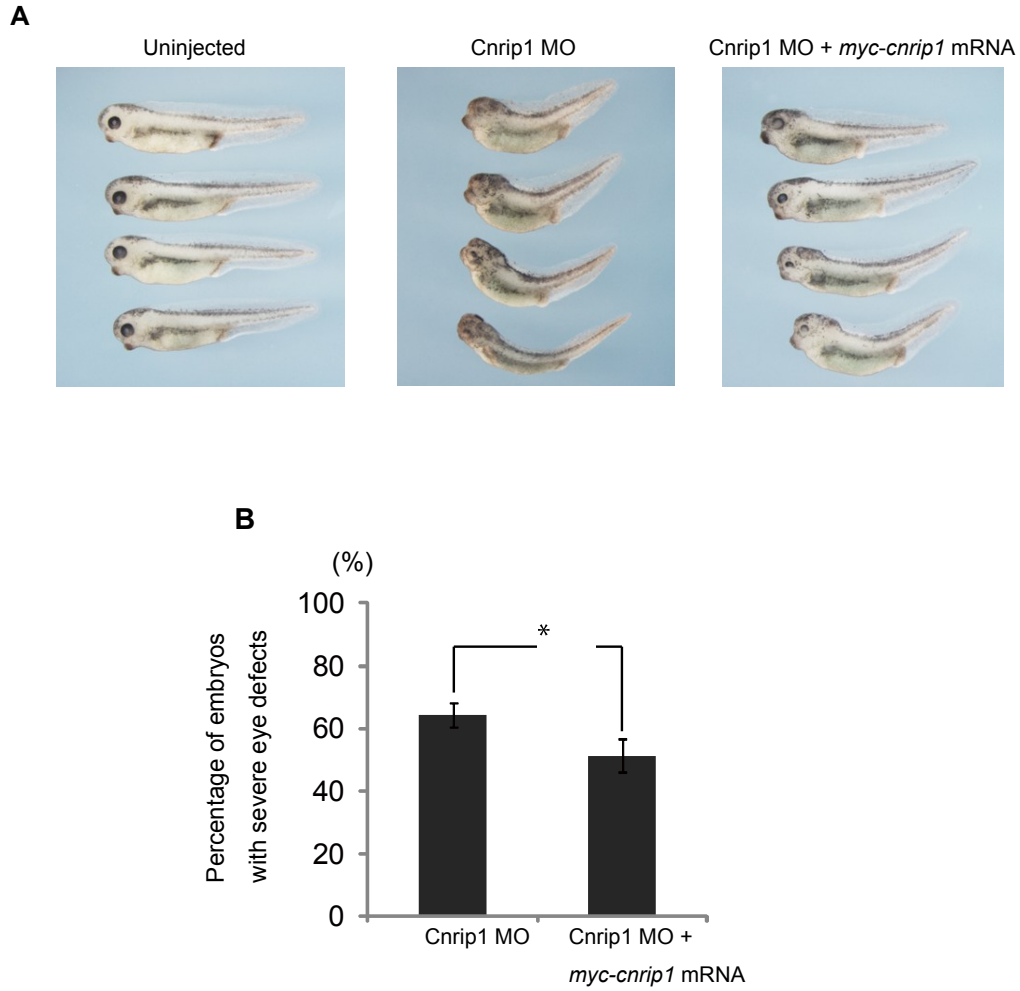

Figure 7. Partial rescue of eye development in *cnrip1* knockdown embryos.

Cnrip1 MO (5 ng) was co-injected with or without *myc-cnrip1* mRNA (350 pg) into the left dorsoanimal blastomere of 8-cell stage embryos. Injected embryos were cultured until stage 37/38. (A) The respective morphologies are shown as lateral views with anterior leftwards and dorsal upwards. (B) Left bar, 64% of embryos injected with cnrip1 MO alone exhibited severe eye defects (absent or very small eyes). Seventy-one embryos were examined in three independent experiments. Right bar, 51% of embryos injected with cnrip1 MO plus *myc-cnrip1* mRNA displayed severe eye defects. Ninety-six embryos were examined in three independent experiments. Values are mean ± SD of the percentages of embryos with severe eye defects from three independent experiments; *, $p<0.05$. “Very small eyes” include small eyes with a dot-like, crescent, or gibbous shape, but not small eyes with a nearly round shape.

Knockdown of *cnrip1* in the D1.1 blastomere causes eye and head defects

In *cnrip1* knockdown experiments mentioned above (Figure 4B, 4C, 5 and 7), cnrip1 MO has been injected at the 4-8 cell stage into the dorsal animal region, which is divided to become the dorsal-most animal blastomere (the D1.1 blastomere) and the dorsolateral animal blastomere (the D1.2 blastomere) at the 16-cell stage (Figure 8A). The progeny cells of the D1.1 and D1.2 blastomeres contribute to eye and neural tissues (Moody, 1987; Huang and Moody, 1993). To determine where *cnrip1* functions, 2.5 ng of cnrip1 MO was injected into the left D1.1 or D1.2 blastomere of the 16-cell stage embryos. As shown in Figure 8B-D, *cnrip1* knockdown in the D1.1 blastomere led to the reduction of eyes and head structure in the left sides, compared with the uninjected right sides. *cnrip1* knockdown in the D1.2 blastomere had little effects on the eye and head development, but caused slightly leftward curved axes (Figure 8E-G). These results suggest that *cnrip1* has distinct roles in distinct blastomeres: *cnrip1* primarily functions in the progeny of the D1.1 blastomere to regulate eye and head development, while it primarily functions in the progeny of the D1.2 blastomere to regulate axial development.

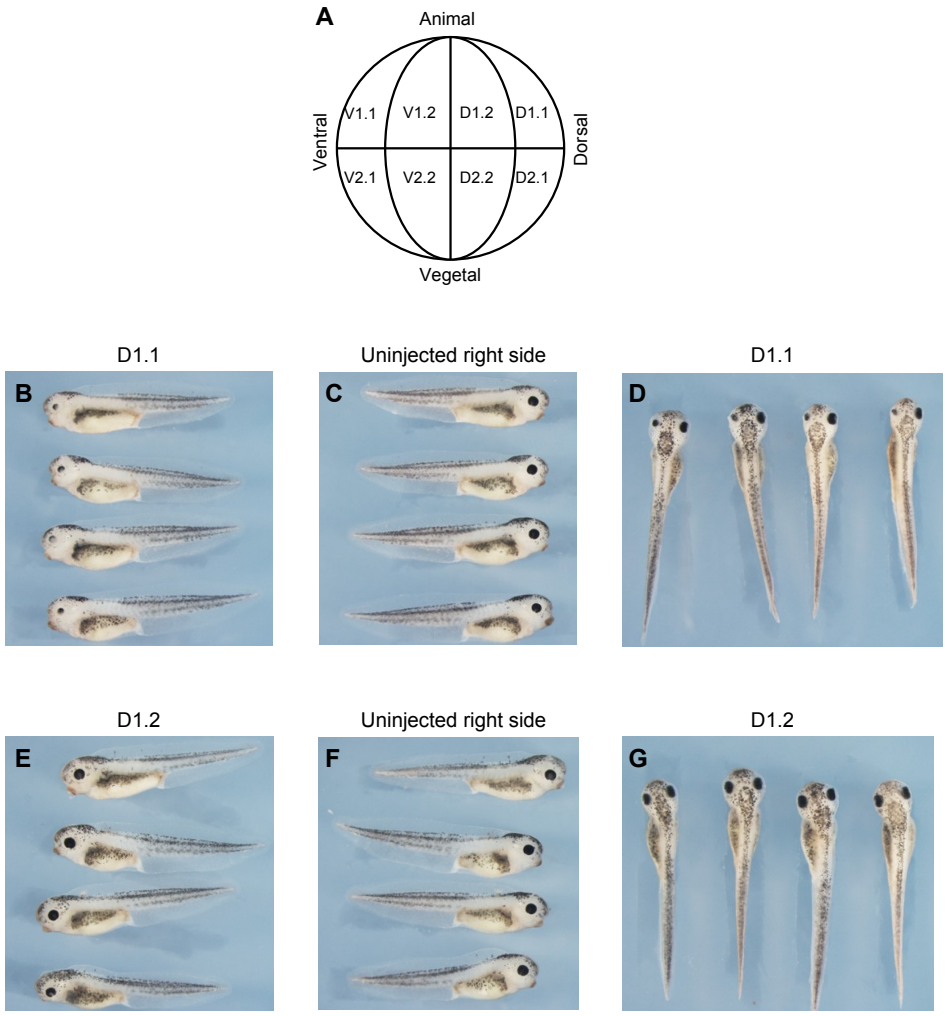

Figure 8. Knockdown of *cnrip1* in the D1.1 blastomere causes eye and head defects.

(A) Schematic diagram of a 16-cell stage *Xenopus laevis* embryo. (B-G) Cnrip1 MO (2.5 ng) was injected into the left D1.1 or D1.2 blastomere at the 16-cell stage, and injected embryos were cultured until stage 41. The uninjected right sides served as controls. The representative results from two independent experiments are shown. (B,C,E,F) Lateral views with dorsal upwards. (D,G) Dorsal views with anterior upwards. (B-D) Embryos injected with cnrip1 MO into the left D1.1 blastomere showed eye and head defects in the left sides (100%, n=40). (C) Normal right eyes were observed in the uninjected right sides. (E-G) Embryos injected with cnrip1 MO into the left D1.2 blastomere showed no significant defects in eyes and heads, but exhibited slight bending of axes to the left (90%, n=40). (F) Normal right eyes were observed in the uninjected right sides.

The expression pattern of *cnr1* in *Xenopus laevis* embryos

To investigate whether *cnr1* is involved in eye and development, the temporal and spatial expression of *Xenopus laevis cnr1* during early developmental stages was analyzed. Unlike *cnrip1*, *cnr1* expression was kept at a relatively low level during gastrula and neurula stages (Figure 9A). The expression level of *cnr1* increased remarkably at stage 25 and reached a maximum at stage 33/34 (Figure 9A). Whole-mount *in situ* hybridization showed that *cnr1* was expressed in the neural plate at stage 19 (Figure 9B). At stage 33/34, *cnr1* was not expressed in the spinal cord or the notochord, but it was expressed in the head (including the brain, eye and facial region) (Figure 9B). Thus, the expression domain of *cnr1* was partially overlapped with that of *cnrip1* (see Figure 3R).

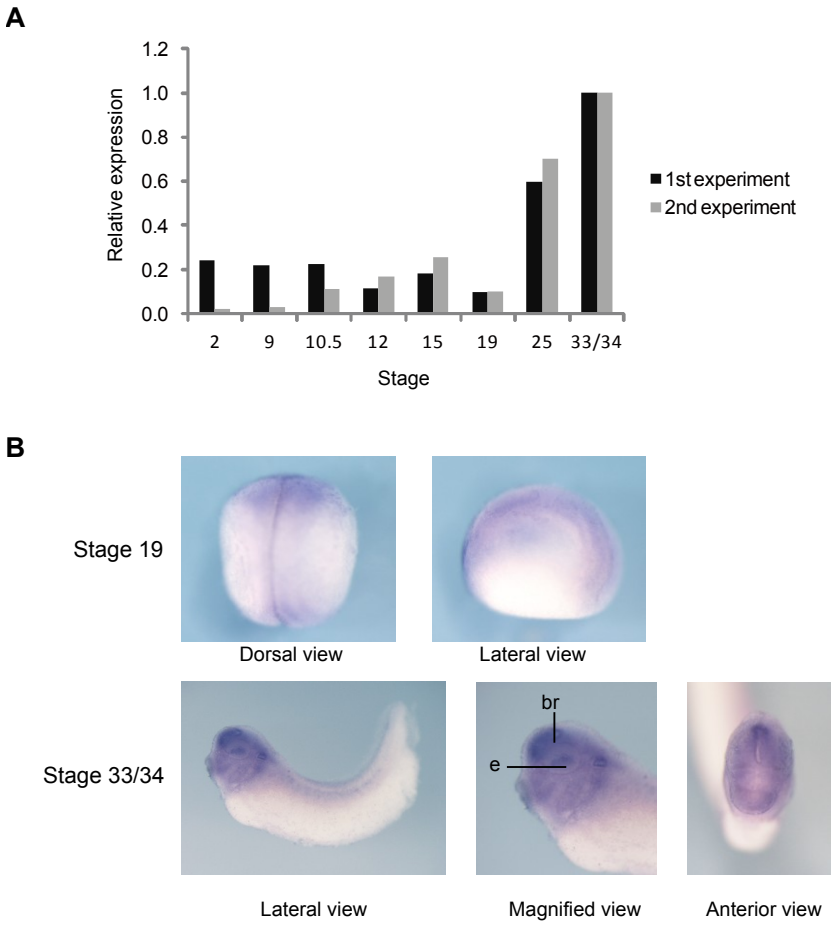

Figure 9. The expression pattern of *Xenopus laevis cnr1* in early development.

(A) qRT-PCR analysis of *cnr1* mRNA expression during *Xenopus laevis* development. The expression level of *cnr1* was normalized to that of *odc*. The bar graph indicates the ratio of the normalized expression level in each stage to that in stage 33/34, from each of two independent experiments. (B) Whole-mount *in situ* hybridization for *cnr1* mRNA. The representative results from two independent experiments are shown. Anterior is upwards in the dorsal view and leftwards in the lateral views. Dorsal is upwards in lateral and anterior views. The lateral view of a stage 33/34 embryo is magnified in the lower middle panel. e, eye; br, brain.

Knockdown of *cnr1* disrupts eye development

To perform *cnr1* knockdown experiment, each of the following MOs was injected into the animal region of two dorsal blastomeres at the 4-cell stage: cnr1 MO1 (targeting the translation initiation site of *cnr1*), cnr1 MO2 (a second non-overlapping MO targeting the 5'UTR region of *cnr1*), and cnr1-5mis MO (a negative control MO with a 5-base mismatch to cnr1 MO1). At stage 41 (late tailbud stage), no obvious defects were observed in the embryos injected with control MO or cnr1-5mis MO, whereas a reduction of the eye was observed in the embryos injected with cnr1 MO1 or cnr1 MO2 (Figure 10A, B).

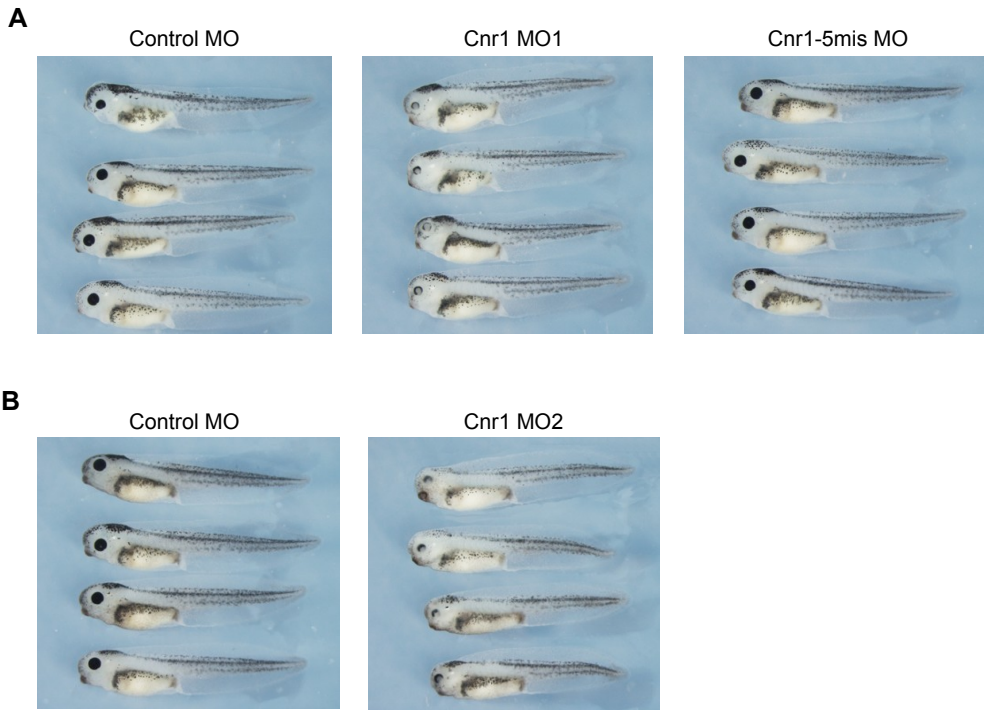

Figure 10. *cnr1* knockdown leads to eye defects.

Control MO, cnr1 MO1, cnr1-5mis MO or cnr1 MO2 (20 ng each/blastomere) was injected into the animal region of two dorsal blastomeres at the 4-cell stage. Injected embryos were cultured until stage 41. Lateral views with anterior leftwards and dorsal upwards. Representative results from three independent experiments are shown. (A) Embryos injected with either control MO or cnr1-5mis MO developed normally (control MO, 100%, n=60; cnr1-5mis MO, 100%, n=60), while those injected with cnr1 MO1 displayed eye defects (100%, n=60). (B) Embryos injected with control MO developed normally (100%, n=60), while those injected with cnr1 MO2 displayed eye defects (100%, n=60).

Effects of *cnr1* knockdown on expression of *sox2*, *otx2*, *pax6* and *rax*

To examine whether *cnr1* knockdown could affect the expression of *sox2*, *otx2*, *pax6* and *rax*, qRT-PCR and whole-mount *in situ* hybridization were performed. A modest reduction of expression of the four marker genes was observed at stages 12.5 and 24, except that the *otx2* expression remains almost unchanged at stage 24 (Figure 11A-D). Thus, the decreases in marker gene expression by *cnr1* knockdown were more modest than those by *cnrip1* knockdown (see Figure 5A-D). Whole-mount *in situ* hybridization result showed that *pax6* expression was mildly suppressed in the cnr1 MO1-injected side at stages 12.5 and 24 (52% and 39%, respectively, Figure 11C). Also, slight reduction in *rax* expression was observed in the cnr1 MO1-injected side at stages 12.5 and 24 (44% and 56%, respectively, Figure 11D). These results indicate that *cnr1* knockdown affects eye development and relevant marker gene expression, similarly to, but more weakly than, *cnrip1* knockdown.

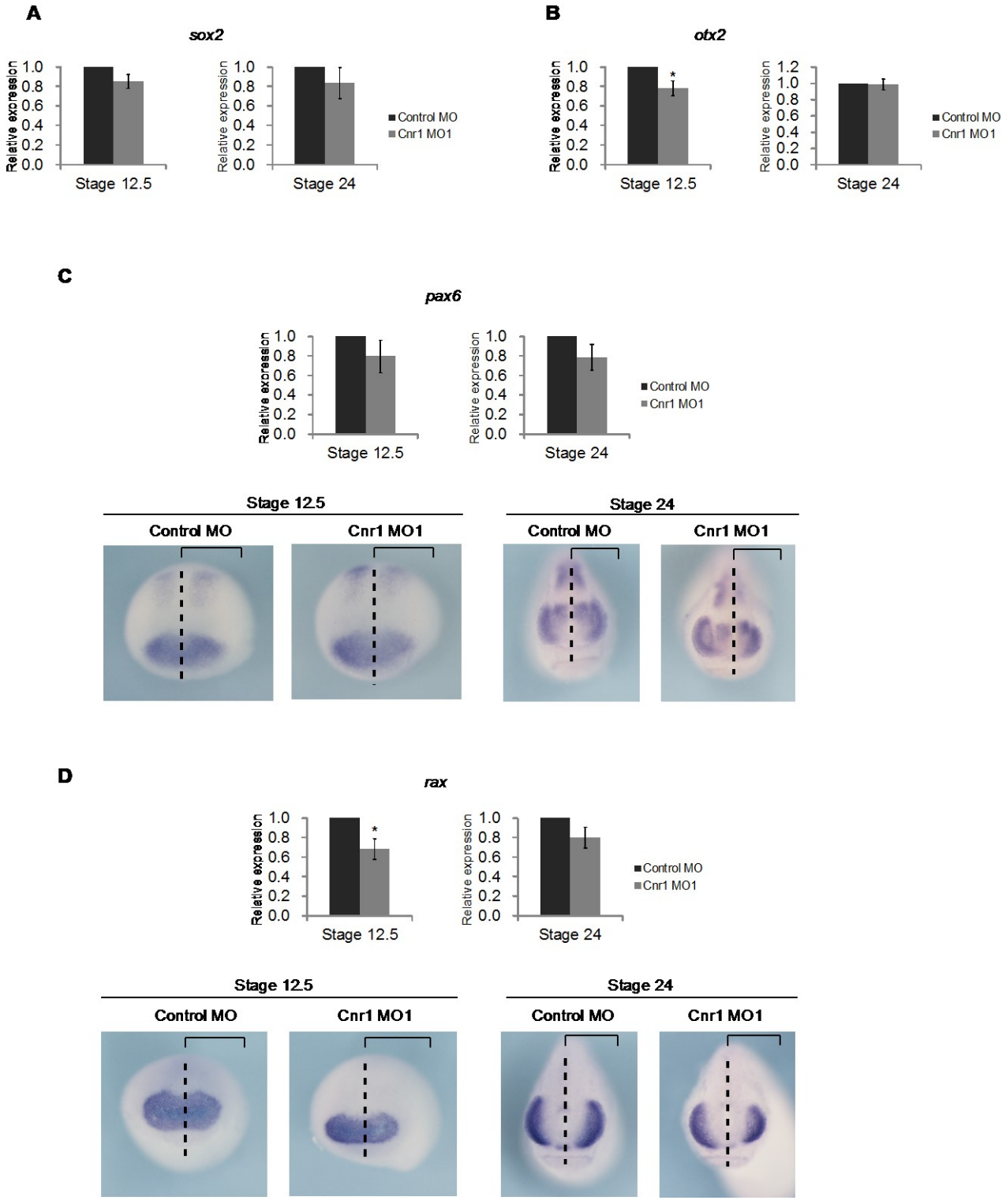

Figure 11. The effects of *cnr1* knockdown on the expression of *sox2*, *otx2*, *pax6* and *rax*.

(A-D) qRT-PCR analysis for the expression of *sox2* (A), *otx2* (B), *pax6* (C) and *rax* (D) in embryos injected with control MO or cnr1 MO1 (20 ng each/blastomere) into the animal region of two dorsal blastomeres at the 4-cell stage. The expression level of each gene at stage 12.5 or

24 was normalized to that of *odc*. Values are mean ± SD of three independent experiments; *, $p<0.05$. (C,D) Whole-mount *in situ* hybridization was performed on embryos injected with 20 ng of control MO or cnr1 MO1 into the animal region of the left dorsal blastomere at the 4-cell stage. The representative results from two independent experiments are shown. Anterior views with dorsal upwards. The black brackets indicate MO-injected left sides. The dashed lines indicate midlines of embryos. (C) A mild reduction of *pax6* expression in the cnr1 MO1-injected side was observed at stage 12.5 (52%, n=21) and stage 24 (39%, n=18). (D) A mild reduction of *rax* expression in the cnr1 MO1-injected side was observed at stage 12.5 (44%, n=18) and stage 24 (56%, n=16).

Discussion

cnrip1 expression is temporally and spatially regulated in *Xenopus laevis* embryonic development

This study has demonstrated the temporal and spatial expression of *Xenopus laevis cnrip1* during early development. *cnrip1* expression was weakly detectable at late blastula stage. Afterwards it was greatly increased and sustained at a high level during gastrula stages, suggesting that the principle function of *cnrip1* initials within the gastrulation period. *cnrip1* was broadly expressed in the animal region of gastrula, where neural and eye induction occur (Zuber et al., 2003; Heasman, 2006). This expression profile is consistent with the result that *cnrip1* knockdown reduced expression of early neural and eye markers (*sox2*, *otx2*, *pax6* and *rax*) in the animal region during the gastrula stage, supporting the conclusion that *cnrip1* is essential for the initial stages of eye and neural development.

In the neural tube of tailbud embryos, *cnrip1* expression was abundant in the alar plate and basal plate, but down-regulated in the vicinities of the roof plate and floor plate. This is similar to the expression pattern of *Xenopus pax6* (Hirsch and Harris, 1997). The obvious change of *pax6* expression in the neural tube by *cnrip1* knockdown and *cnrip1* overexpression was also observed. It can be speculated that *cnrip1* is functionally associated with *pax6* in the later patterning within the neural tube.

At stage 25 (the early tailbud stage), *cnrip1* was highly expressed in notochord, with level being high in the anterior and low in the posterior. However, at stage 33/34 (the late tailbud stage), *cnrip1* expression was eliminated at the rostral and only visible at the

tail tip. Because differentiation of notochord cells proceeds in an anterior to posterior direction during tailbud stages (Nieuwkoop and Faber, 1967), it can be speculated that *cnrip1* may be involved in notochord differentiation. The curved body axis caused by cnrip1 MO may be partly due to developmental defects in the notochord. Further studies will be required to confirm the hypothesis.

cnrip1 is required for eye and neural development

In *Xenopus* embryos, at mid-gastrula stage, the presumptive forebrain is specified in the anterior neuroectoderm by *otx2* expression (Zuber et al., 2003). Eye field specification occurs within the presumptive forebrain domain at late gastrula stage (Zuber et al., 2003). Eye field is characterized by Otx2 and other eye field transcription factors such as Pax6 and Rax (Zuber et al., 2003). Otx2 has no effect on inductions of *pax6* and *rax* expression, but can prevent the inhibitory environment for their inductions (Zuber et al., 2003). Loss of function of any of these three genes results in eye defects, and overexpression of *pax6* or *rax* leads to ectopic eye formation (Andreazzoli et al., 1999; Chow et al., 1999; Zuber et al., 2003; Bailey et al., 2004). *cnrip1* knockdown caused remarkable loss of *pax6* and *rax* expression in the eye field at stage 12.5, suggesting a perturbation of the eye field induction. However, knockdown of *cnrip1* showed mild inhibition on expression of *otx2*, suggesting that the Cnrip1-mediated regulation of *pax6* and *rax* expression may be partially dependent on Otx2. There is also another possibility that such regulation is Otx2-independent. Of interest, the reduction of *rax* expression by cnrip1 MO at stage 12.5 was more severe than that at stage 24, suggesting a compensatory effect that recovers the expression at the later stage. Conversely, overexpression of *cnrip1* was able to expand expression of *pax6* and *rax*. These data

taken together suggest an instructive role of *cnrip1* in eye development.

At the early gastrula stage when neural induction occurs (before eye field induction occurs) (Zuber et al., 2003; Heasman, 2006), the expressions of the major pan-neural marker *sox2* and the major anterior marker *otx2* were markedly decreased by *cnrip1* knockdown. At the late gastrula stage, reduction of *sox2* expression by *cnrip1* knockdown was more remarkable in the anterior neural plate than that in the posterior neural plate (see Figure 5A, lowermost right). The first morphological defect due to *cnrip1* knockdown was observed in the anterior neural tube closure at late neurula stage. And at later stages, head defects caused by *cnrip1* knockdown were observed. These results suggest that *cnrip1* globally affects the early neural development, especially the anterior neuroectoderm. Thus *cnrip1* is essential for early neural development as well as eye development.

cnrip1 may be associated with both *cnr1*-dependent and *cnr1*-independent pathways in eye and neural development

cnrip1 expression was relatively high at gastrula stages and late neurula stages, while *cnr1* expression was kept at a relatively low from gastrula to neurula stages. This suggests a possibility that the developmental role of *cnrip1* is independent on *cnr1,* at least during gastrula stages. However from the tailbud stage, the expression of both of *cnrip1* and *cnr1* was significantly increased. Whole-mount *in situ* hybridization showed that the expression domain of *cnrip1* was partly overlapped with that of *cnr1* in the neural plate at neurula stage and in the head (including eyes) at the tailbud stage, suggesting a possibility of functional interaction between CNRIP1 and CNR1 in the later neural development. At the tailbud stage, the eye and head defects induced by

cnrip1 MO were more severe than those induced by cnr1 MOs, although the dose of cnrip1 MO was only half of those of cnr1 MOs. Furthermore, the suppression in the expression of the neural and eye marker genes by cnrip1 MO was more remarkable than that by cnr1 MO. It seems that *cnrip1* plays more important role than *cnr1* in eye and neural development. However, the eye defects and the downregulation of eye marker genes induced by cnrip1 MO were partially similar to those induced by cnr1 MO, suggesting a possibility that the developmental role of *cnrip1* is partly dependent on *cnr1*. Identification and characterization of endogenous Cnrip1-binding partners in future may define detailed molecular mechanisms underlying the developmental role of Cnrip1.

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Genes to Cells, in press.

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