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Molecular Cloning of the Genes Encoding the PR55/B β / δ Regulatory Subunits for PP-2A and Analysis of Their Functions in Regulating Development of Goldfish, *Carassius auratus*

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Abstract: The protein phosphatase-2A (PP-2A), one of the major phosphatases in eukaryotes, is a heterotrimer, consisting of a scaffold A subunit, a catalytic C subunit and a regulatory B subunit. Previous studies have shown that besides regulating specific PP-2A activity, various B subunits encoded by more than 16 different genes, may have other functions. To explore the possible roles of the regulatory subunits of PP-2A in vertebrate development, we have cloned the PR55/B family regulatory subunits: β and δ , analyzed their tissue specific and developmental expression patterns in Goldfish (*Carassius auratus*). Our results revealed that the full-length cDNA for PR55/B β consists of 1940 bp with an open reading frame of 1332 nucleotides coding for a deduced protein of 443 amino acids. The full length PR55/B δ cDNA is 2163 bp containing an open reading frame of 1347 nucleotides encoding a deduced protein of 448 amino acids. The two isoforms of PR55/B display high levels of sequence identity with their counterparts in other species. The PR55/B β mRNA and protein are detected in brain and heart. In contrast, the PR55/B δ is expressed in all 9 tissues examined at both mRNA and protein levels. During development of goldfish, the mRNAs for PR55/B β and PR55/B δ show distinct patterns. At the protein level, PR55/B δ is expressed at all developmental stages examined, suggesting its important role in regulating goldfish development. Expression of the PR55/B δ anti-sense RNA leads to significant downregulation of PR55/B δ proteins and caused severe abnormality in goldfish trunk and eye development. Together, our results suggested that PR55/B δ plays an important role in governing normal trunk and eye formation during goldfish development.

Keywords: protein phosphatase, PP-2A, PR55/B β / δ , eye, lens, gene expression, developmental regulation, phosphorylation

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Introduction

The reversible phosphorylation of proteins is an important posttranslational modification in eukaryotes¹⁻³ and an essential mechanism regulating functions of more than 30% total cellular proteins.^{4,5} The protein phosphatase-2A (PP-2A) is one of the major phosphatases in eukaryotes, contributing to more than 50% serine/threonine phosphatase activity and participating in many cellular processes such as signal transduction, gene expression, neurotransmission, cell cycle control, cell transformation and senescence.¹⁻⁷ In this regard, our recent studies have indicated that PP2A is associated with carcinogenesis^{8,9} and is highly regulated in ocular tissues.¹⁰⁻¹²

The holoenzyme of PP-2A is a heterotrimer, consisting of a scaffold A subunit, a catalytic C subunit and a regulatory B subunit.¹³⁻¹⁶ While the A and C subunits exist in two isoforms encoded by different genes, the B subunits exist in approximately 26 different isoforms and are encoded by four subfamilies of genes (B or PR55, B' or PR61, B'' or PR72, and B''' or PR93/PR110) and each family consists of multiple genes, with each gene generating multiple splice variants.^{13,14} These B subunits exhibit differential subcellular localization as well as tissue-specific and developmentally-regulated expression patterns. Variations in their expression pattern and cellular localization of B subunits provide substrate specificity, which is thought to be the molecular basis for the appropriate regulation of numerous cellular processes.^{1,2,5-7,13,14}

The major function of the regulatory subunits for PP-2A is to provide specific PP-2A activity in different cellular compartments and different tissues of organisms.^{1,2,5-7,13,14} In addition, these different regulatory subunits may have other functions independent of PP-2A. For example, SG2NA, a member of the B''' or PR93/110 family, has been shown to act as a molecular scaffold to promote localization of the estrogen receptor to the plasma membrane and organize the ER-eNOS membrane signaling complex in endothelial cells.¹⁷ In addition, it is found that the *Drosophila* orthologue member of SG2NA named CKA can form a physical complex with several kinases including HEP, BSK, and components of AP-1 family including JUN and FOS.^{18,19} To further explore the independent functions of the regulatory subunits of PP-2A, we have cloned two members of the PR55/B family from goldfish, established their tissues specific and

developmental expression patterns. Moreover, we have designed antisense expression construct to block translation of the δ isoform and demonstrated that injection of the anti-sense RNA from PR55/B δ significantly downregulates the expression of this regulatory subunit at several developmental stages. Furthermore, inhibition of PR55/B δ expression via anti-sense RNA-mediated blockage of translation caused severe phenotype of the developing goldfish embryos including microphthalmia (small eye) and abnormal trunk. Thus, our results demonstrate that the PR55/B δ plays an important role in regulating vertebrate organogenesis.

Materials and Methods

Animals

The goldfish samples at the age of 6 months to one year were collected from the Experimental Fish Culture Facility of the Key Laboratory of the Educational Ministry of China in Hunan Normal University. And the fertilization was conducted at the laboratory.

Chemicals

The RNA extraction kit was purchased from Omega, the reverse transcription kit from Invitrogen, Inc, the protein size marker from Fermentas. The 5' and 3' RACE cloning kit was obtained from the Clontech, Inc. The PCR Taq polymerase and the PMD18-T vector were purchased from Takara Inc. The antibodies used for this study were purchased from Santa Cruz Biotechnology and from Sigma, Inc. Gel purification kit and all the oligo primers were provided by Sangon, Inc.

Collection of tissues and embryos

Goldfish were sacrificed through removal of the gill tissue. Various tissues including liver, spermary, ovary, brain, kidney, heart, muscle, gill and fin were quickly dissected out on ice and then frozen under liquid nitrogen for homogenization, first with a mortar and then with 1 ml syringe (18.5 G and 23.5 needles passed). Artificial fertilization was conducted in Hoff's solution (0.1 g CaCl₂, 0.05 g KCl, 3.5 g NaCl dissolved in 1000 ml distilled H₂O). The fertilized egg membranes were removed with 0.4% pancreatic protease and the de-membrated eggs were allowed to develop at 22 °C in Hoff's solution. Under microscopic examination, the developing embryos at stages of 2-cell,



multiple-cell, blastula, gastrula, neurula, optical vesicle, brain differentiation, muscle differentiation, heart beat, eye pigmentation, body pigmentation and hatching larvae were collected and frozen under liquid nitrogen. The frozen embryos were homogenized for extraction of total RNA and proteins as described below.

Molecular cloning of the PR55/B family of PP2A

The two cDNAs for PR55/B β / δ were cloned using 5'-RACE and 3'-RACE as previously described.^{9,20} Briefly, the specific primers used to clone these cDNAs were designed using Jellyfish and prime 5.0 softwares and were shown in Table 1. The homology-based reverse transcriptase-polymerase chain reaction (RT-PCR) cloning was used to isolate partial B subunit cDNAs from total adult goldfish brain RNA. Additional 5' sequences for B subunits were obtained by 5' rapid amplification of cDNA ends (5'-RACE) from goldfish brain RNA according to instructions supplied with the Marathon cDNA amplification kit (Clontech, Inc.). 3' Race was performed using 3'-RACE kit.

Reverse transcription-linked polymerase chain reaction (RT-PCR)

The reverse transcription was conducted with a kit from Invitrogen (Invitrogen #18085-019) as previously described.^{8,9,20-22} Briefly, 2 μ g of total RNA were used in a total reaction volume of 20 μ l and 2 μ l of the reverse transcription reaction mixture were used for PCR reaction. To detect the mRNA expression of PR55/B β / δ , three pairs of specific primers as well as the β -actin primers (Table 1) were used. For PCR amplification, both specific primers and β -Actin primers were added into the same reaction at the beginning of PCR, and the PCR reaction was continued 30 cycles. At the end of each reaction, the PCR products were separated by agarose gel (1.5%) electrophoresis and photographed under UV illumination.

Western blot analysis

Western blot analysis was conducted as previously described.^{8-10,23} Briefly, 50 or 100 μ g of total proteins from various tissues and each developmental stage of embryos were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred into supporting nitrocellulose membranes (Bio-Rad). The protein blots were blocked with 5% milk in TBS (10 mM Tris, pH 8.0; 150 mM NaCl) for 60 minutes at normal room temperature. Then, each blot was incubated with the anti-B55 β / δ antibodies (Santa Cruz Biotechnology) at a dilution of 1:100 in 5% milk prepared in TBS overnight at 4 °C with mild shaking. After washing 3 times with TBS-T (TBS with 0.05% Tween-20), 15 minutes for each, the blot was incubated with a secondary antibody (anti-rabbit IgG from Santa Cruz Biotechnology) at a dilution of 1 to 1000 for 45 minutes. After washing twice with TBS-T and once with TBS (15 minutes each), the PR55 proteins were detected with an enhanced chemiluminescence detection kit according to the instruction manual from Amersham.

As reference, after stripping the previous antibody, the blot was re-hybridized with the anti- β -actin primary antibody (1:2000 from Sigma, Inc.). After washing with TBST 3 times, the blot was incubated with the anti-mouse IgG (secondary antibody from GE Health Care, Inc. diluted in 1:1000). After washing with TBST twice times and TBS one time, the β -actin level was detected as described above.

Quantitation of RT-PCR and Western blot results

After RT-PCR, the relative density of each specific band verse β -actin control band was quantitated as described before.²⁴ Both RT-PCR and Western blot results in the x-ray films were analyzed with the Automated Digitizing System from the Silk Scientific Corporation. The relative expression levels (fold) were calculated by dividing the total pixel from each band under investigation by the total pixel from the corresponding β -actin band. The quantitative data

Table 1. Oligo primers used for RT-PCR analysis to detect expression of PR55/B β / δ .

| | Forward primer | Reverse primer |
|----------------|------------------------------|-----------------------------|
| PR/B β | 5'-CCCCAGTAATCGCTCCTTCT-3' | 5'-AACTTTGCGTGGCTTGAGAAT-3' |
| PR/B δ | 5'-CGCATCAACCTGTGGCACTT-3' | 5'-GGTCTCAACGGGTCGATTCTC-3' |
| β -actin | 5'-CCGTGACCTGACTGACTACCTC-3' | 5'-ATACCGCAAGATTCCATACCC-3' |



averaged from three independent experiments and statistics were analyzed by students' t-test.

Preparation of antisense expression construct for PR55/B δ

The full-length cDNA for PR55/B δ was cloned into the pEGFP vector in a reverse direction so that the anti-sense strand will be expressed under the direction of the viral promoter as previously described.²⁵ The pEGFP vector alone was used as mock.

Injection of plasmids and observation of the injected embryos development

Both vector and anti-sense expression vector were amplified in DH-5 α , and then purified through maximal plasmid purification kit (Qiagen) according to the instruction manual. The 500 ng of purified plasmids in 0.05 μ l were injected into each fertilized egg using a microinject developed by Shanghai Instrument, Inc. The vector-injected embryos and the anti-sense expression construct-injected embryos were allowed to develop at 22 °C in Hoff's solution. The wound embryos were removed from the experiments. The phenotypes of each developmental stage were recorded with microscopy (Table 2).

Results

Molecular cloning of the PR55/B β / δ cDNAs of PP-2A from goldfish

Using a molecular strategy as previously described,^{9,20} the full-length cDNAs for PR55/B β and PR55/B δ were isolated. These cDNA sequences were deposited to gene bank database with the access numbers of FJ356012 and FJ356011 for PR55 β and PR55 δ , respectively.

Sequence analysis revealed that the full-length PR55/B β cDNA consists of 1940 bp, with an open reading frame of 1332 nucleotides coding for a deduced

protein of 443 amino acids (Fig. 1A). Amino acid sequence alignment showed that goldfish PR55 β shares high levels of identity to those from African clawed frog (88.4%), mouse (92.2%) and human (92.5%) (Figs. 1B and 3B). The full-length PR55/B δ cDNA contains 2163 bp with an open reading frame of 1347 nucleotides, which encodes a deduced protein of 448 amino acids (Fig. 2A). The amino acid sequence alignment demonstrated that the goldfish PR55 δ protein shared a sequence identity of 98.4%, 87.7%, 86.9%, 86.9% and 86.9% with those from zebrafish, western clawed frog, chicken, mouse and Norway rat, respectively. (Figs. 2B and 3C).

Analysis of the amino acid sequence in the deduced protein PR55/B β / γ / δ of PP-2A through both ExpASY and the conserved domain architecture retrieval tool (DART) revealed presence of the WD-40 tandem repeats in all three isoforms (Boxed regions in Figures 1A and 2A, and data not shown), indicating the functional importance for their binding to the scaffold subunits of PP-2A.^{12,15,16,21,22,26} Moreover, an 80.8% of sequence identity between PR55/B β and PR55/B δ were found (Fig. 3A). In addition, the N-termini in PR55/B β and PR55/B δ are significantly diversified (Fig. 3A).

Tissue specific expression of PR55/B family members

To explore the potential functions of PR55/B family members in various tissues of the lower vertebrates, we examined the mRNA levels for PR55/B β / δ of PP-2A in liver, spermary, ovary, brain, kidney, heart, muscle, gill and fin from goldfish using reverse transcription-linked polymerase chain reaction (RT-PCR) analysis. As shown in Figure 4A, a band of 370 bp cDNA was amplified using specific primers for PR55/B β in two tissues: high level of expression in brain and low level of expression in heart. Similarly, a single band of 372 bp was amplified in all tissues examined for PP2A/B δ (Fig. 4B). Among these tissues, brain, ovary and kidney contained the highest levels of PR55/B δ mRNA expression (Fig. 4B). In comparison with brain, ovary and kidney, muscle and heart displayed a slight decrease in PR55/B δ mRNA expression, and fin, gill, spermary and liver showed further decrease.

To further explore the tissue-specific distribution of PR55/B β / δ , we conducted western blot analysis. As shown in Figure 5A, PR55/B β protein was

Table 2. Statistical results of abnormality of development derived from inhibition of P55/B δ expression from four independent experiments.

| Injection group | Total injected fertilized eggs | Reduction of eye size |
|-------------------------------|--------------------------------|-----------------------|
| pEGFP-C3 (vector) | 100% (126) | 6.2% \pm 2% |
| pEGFP-antisense-P55/B β | 100% (128) | 80.2% \pm 6.6% |



detected at relatively high level in the brain tissue but much attenuated in the heart. All other tissues have no detectable PR55/B β protein. In contrast to PR55/B β , the PR55/B δ was highly expressed in the brain and heart, moderately expressed in liver, spermary, ovary, muscle, fin and gill, and the lowest level detected in kidney (Fig. 5B).

Developmental expression patterns of PR55/B β / δ

To explore the possible function of the PR55/B β / δ during goldfish development, we first determined their developmental expression patterns at both mRNA (Fig. 6) and protein (Fig. 7) levels. Through RT-PCR analysis, we demonstrated that PR55/B β mRNA

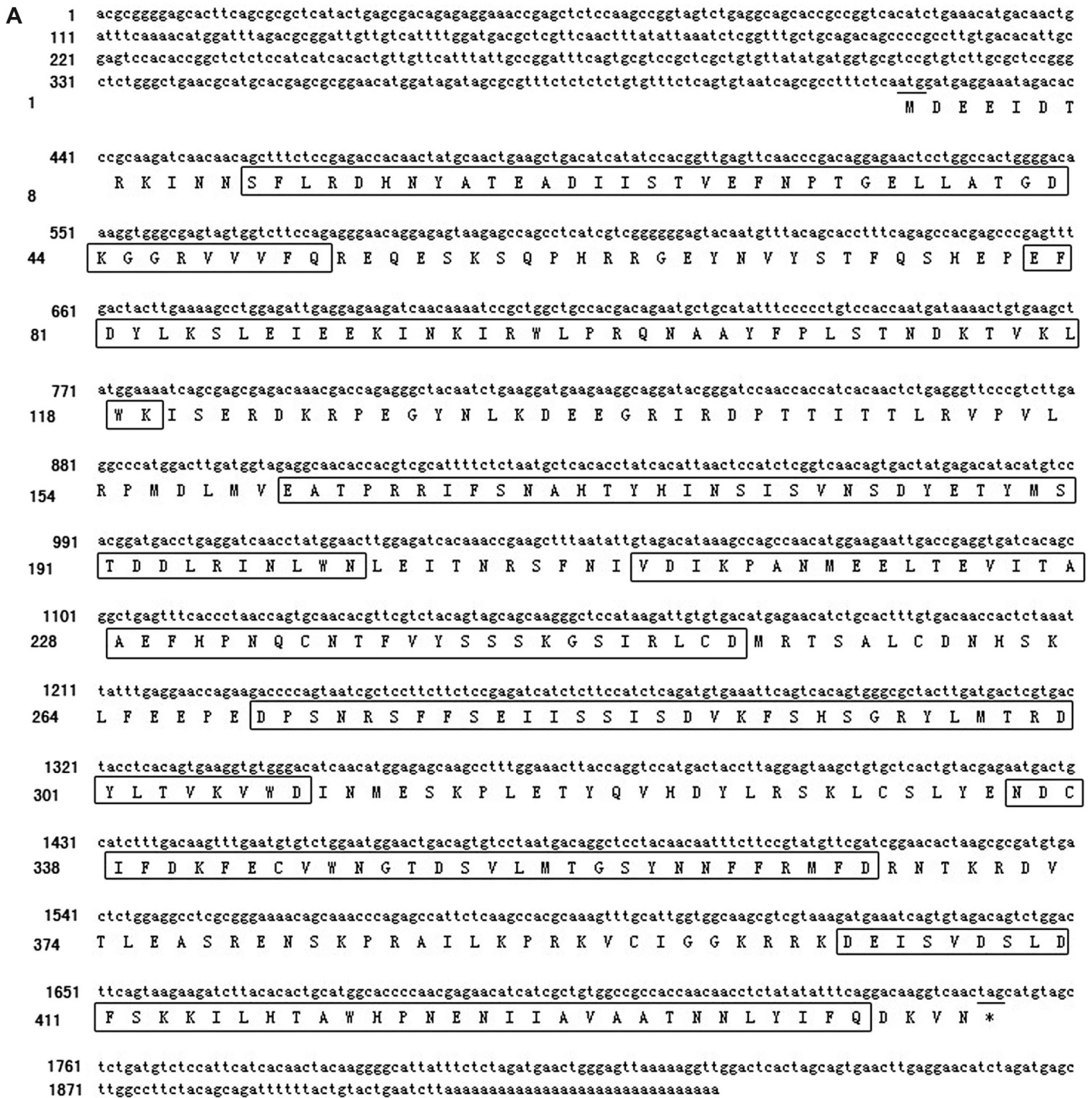


Figure 1. (Continued)

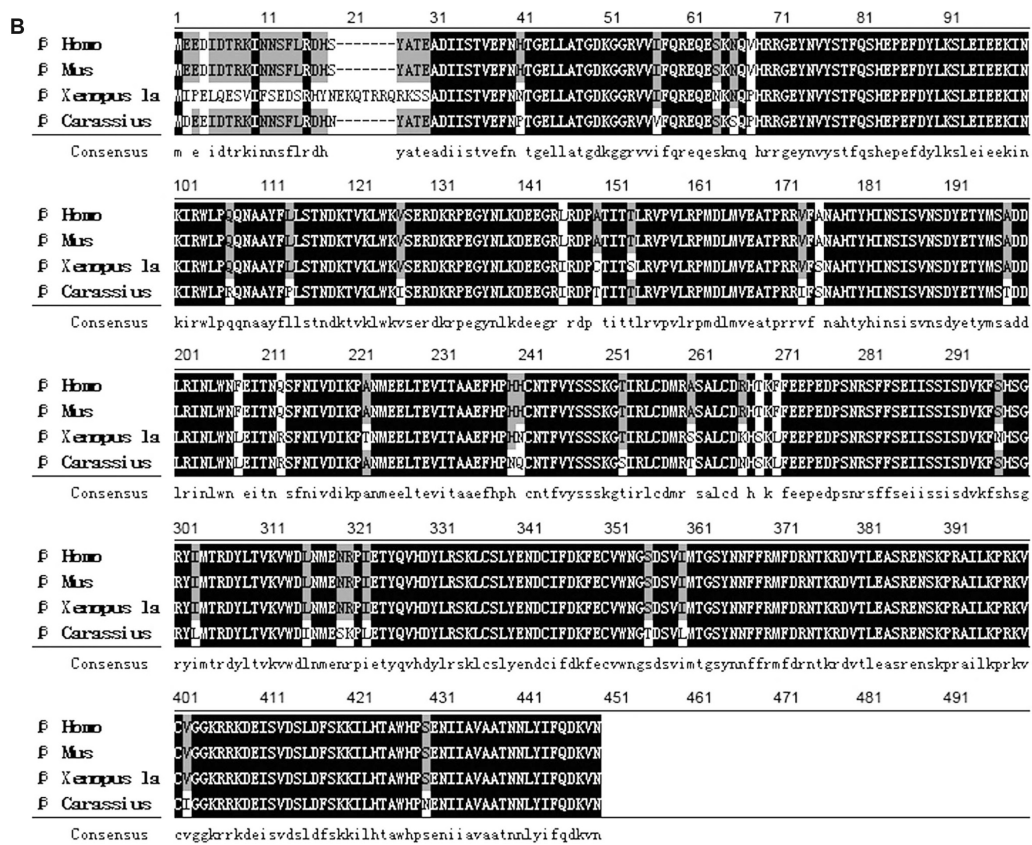


Figure 1. A) The full length cDNA of PR55/B β for PP-2A and the deduced protein sequences. The initiation and termination codons are underlined. The predicted amino acid sequence is shown in the one-letter code below the nucleotide sequences. The WD-40 repeats were high-lighted by open box. **B)** Alignment of the deduced amino acid sequence from goldfish PR55/B β of PP-2A with the known PR55/B β sequences of PP-2A from human (*Homo sapiens*, NM_004576), mouse (*Mus musculus*, NM_088979) and frog (*Xenopus laevis*, BC130184).

level was relatively low from two-cell, multiple-cell to blastula stage. This mRNA level was substantially increased at the gastrula stage transiently, then dropped down at the neurula stage. From the optic vesicle, through brain and muscle differentiation, to heart beat, the PR55/B β mRNA became gradually increased. And it maintained relatively stable at this level in the next four different stages of development (Fig. 6A). Different from the expression pattern of PR55/B β mRNA (Fig. 6A), the PR55/B δ mRNA, in the very first three stages of development, displayed the highest level, then slightly dropped down from gastrula to neurula stages, gradually increased from optical vesicle stage to brain differentiation stage, maintained at this level at muscle differentiation and heart beat stages, and became gradually decreased from eye pigmentation, to hatching larval stages (Fig. 6B).

To further confirm the developmental expression of PR55/B β / δ at the protein level, we have conducted western blot analysis. As shown in Figure 7A,

PR55/B β protein seems to be undetectable at any stage of development. In contrast, the PR55/B δ protein was maintained at similar levels in the 8 different stages examined: multiple-cell, blastula, gastrula, neurula, optic vesicle, brain differentiation, eye pigmentation, body pigmentation and hatching (Fig. 7B).

Attenuation of PR55/B δ protein expression led to severe abnormality in eye development of goldfish

To further confirm the role of PR55/B family subunit in regulating development of goldfish, we constructed an expression construct for the generation of the anti-sense strand RNA from the PR55/B δ cDNA. Basically, the full length cDNA of PR55/B δ was ligated into the pEGFP-C3 vector in the non-coding direction so that the anti-sense RNA would be generated when injected into fertilized eggs. The empty vector was used as mock injection. Expression of the antisense PR55/B δ RNA substantially attenuated the translated level of PR55/



Bδ protein at several developmental stages examined (Figs. 8A and 8B). When PR55/Bδ protein level was significantly downregulated, the development of the goldfish displayed severe phenotype in both trunk and eye (Fig. 8D) in comparison with those in the normal larvae (Fig. 8C). The trunk was severely bent and the eye appeared in much small size (microphthalmia) (Fig. 8D Table 2) in comparison with the vector-injected embryos (Fig. 8C). Thus, our results demonstrate that PR55/Bδ is important

for goldfish organogenesis, especially the trunk and the eye.

Discussion

In the present study, we have demonstrated: 1) The goldfish PR55/Bβ/δ cDNAs contain ORFs of 1332 bp and 1347 bp, coding for the deduced proteins of 443 PR55/Bβ and 448 PR55/Bδ amino acids, respectively; 2) the deduced goldfish PR55/Bβ protein share an amino acid identity of 88.4%, 92.5% and 92.5% to

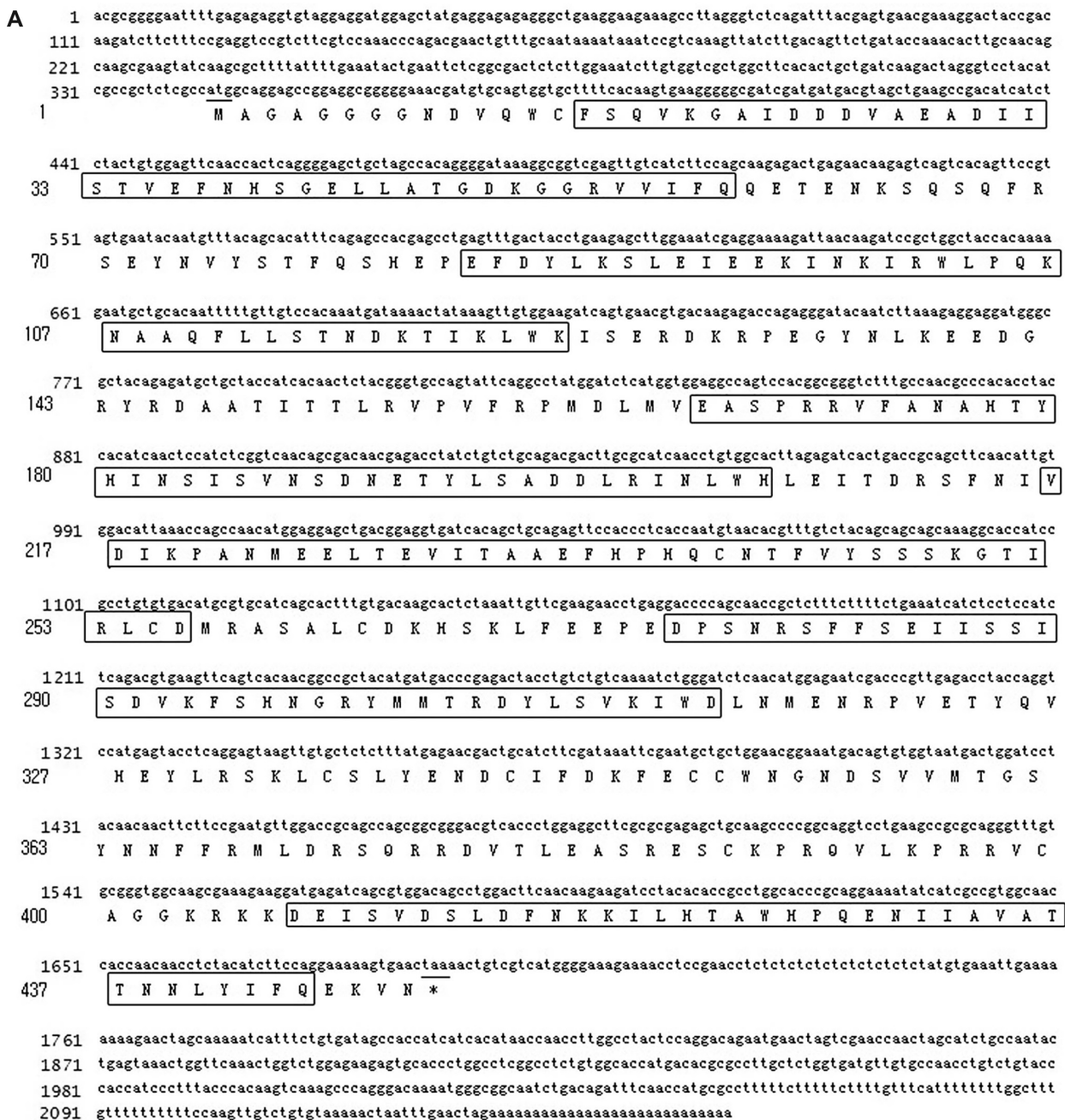


Figure 2. (Continued)

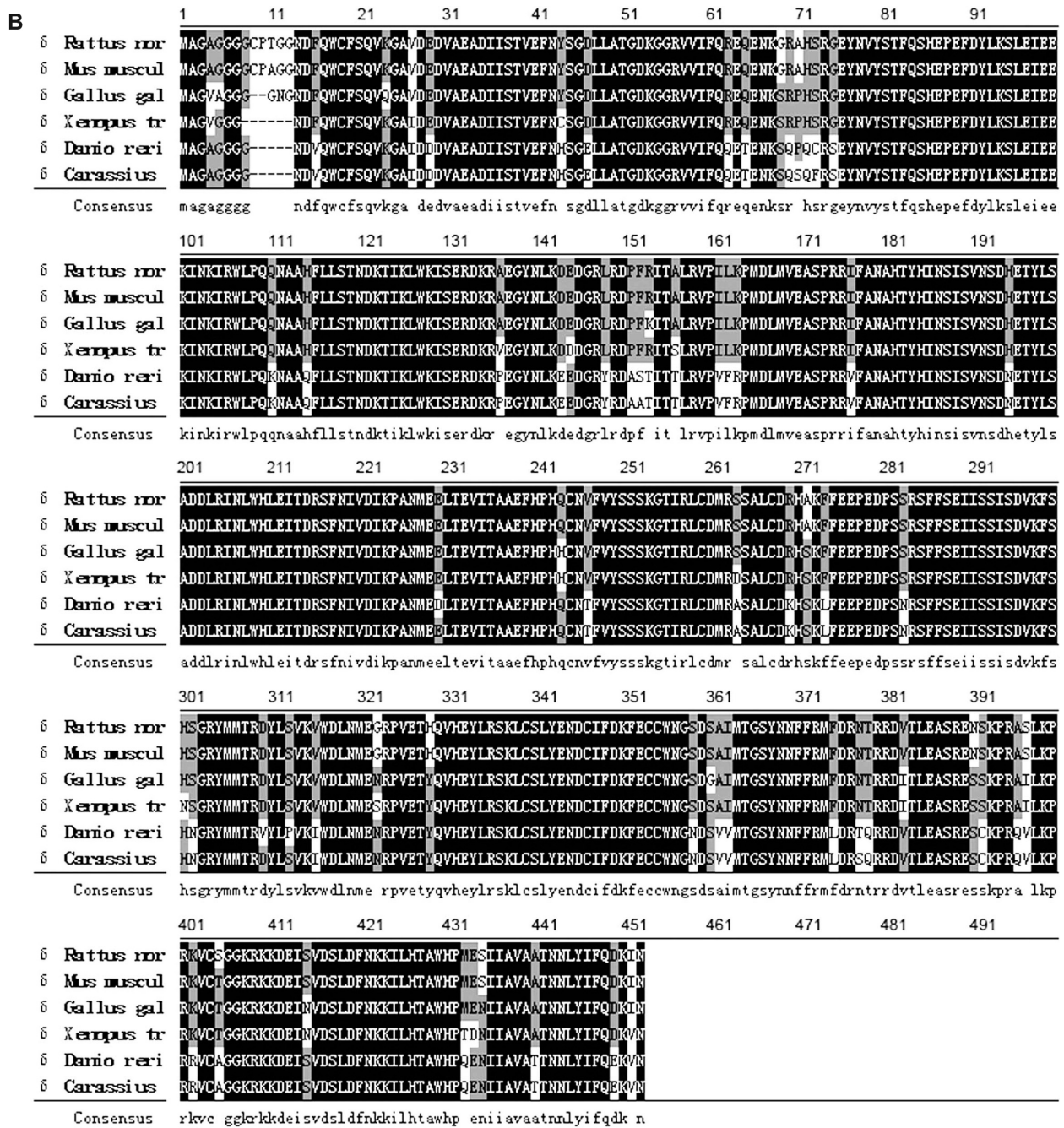


Figure 2. A) The full length cDNA of PR55/B δ for PP-2A and the deduced protein sequences. The initiation and termination codons are underlined. The predicted amino acid sequence is shown in the one-letter code below the nucleotide sequences. The WD-40 repeats were high-lighted by open box. **B)** Alignment of the deduced amino acid sequence from goldfish PR55/B δ of PP-2A with the known PR55/B δ sequences of PP-2A from Rat (*Rattus norvegicus*, NM_144746), Mouse (*Mus musculus*, NM_026391), Chicken (*Gallus gallus*, NM_001006507), Frog (*Xenopus tropicalis*, NM_001006696), and Zebrafish (*Danio rerio*, NM_199776).

that from frog, mouse and human, respectively; the deduced goldfish PR55/B δ protein share an amino acid identity of 98.4%, 87.7%, 86.9%, 86.9% and 86.9% to that from zebrafish, frog, chicken, mouse and rat, respectively; 3) the PR55/B β mRNA is present in brain and heart only, and the PR55/B δ

mRNA is detected in all 8 tissues examined; 4) At the protein level, the PR55/B β and PR55/B δ also displayed distinct patterns; 5) Inhibition of PR55/B δ translation through anti-sense RNA blockage caused severe phenotype of the injected embryos including severe reduction of the eye size. Together, our results

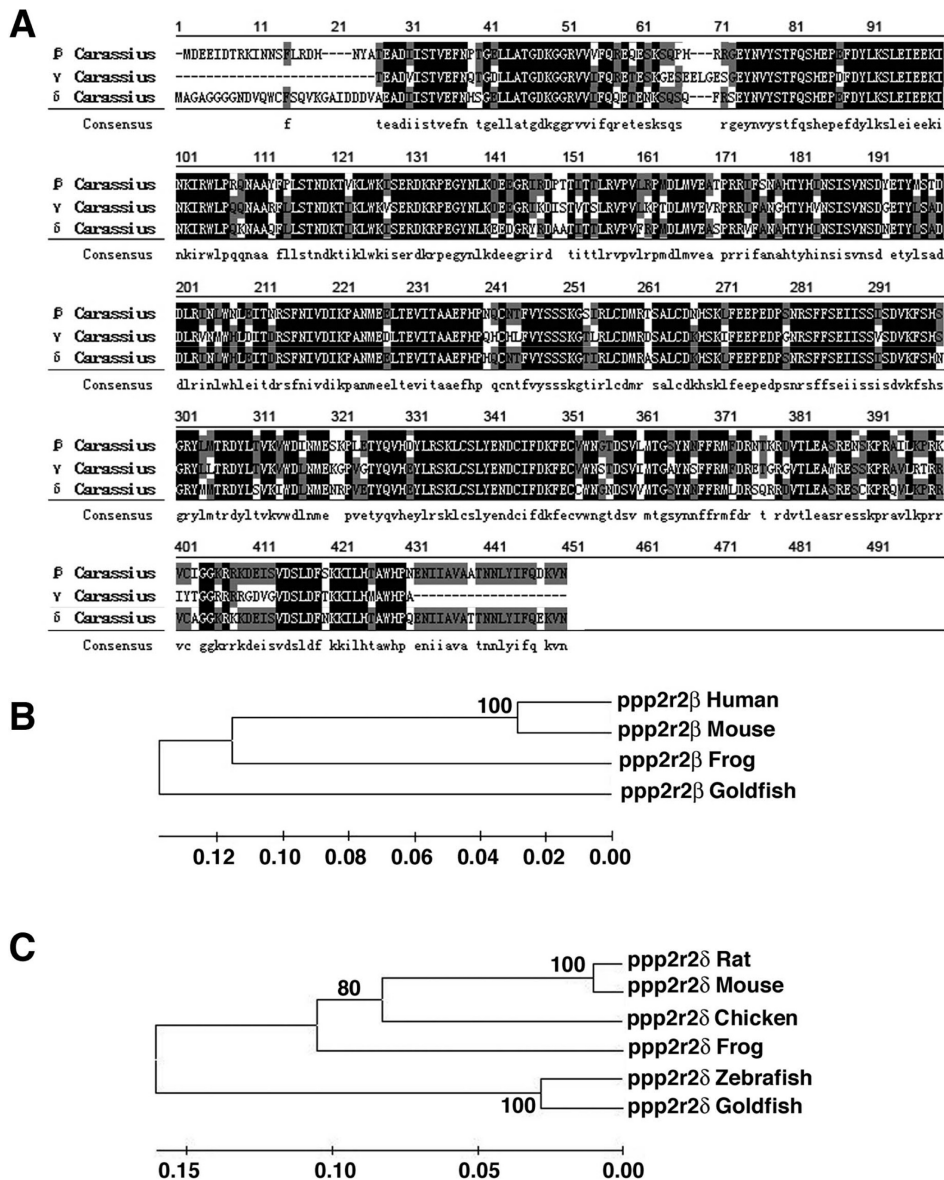


Figure 3. **A**) Amino acid sequence alignment of the PP2A-PR55/B family members, $\beta/\gamma/\delta$ in goldfish (The partial amino acid sequence for PR55/B β is non-published data from Zhao et al). The completely conserved region among the three isoforms is marked by black shadow. The less conserved region is marked by grey shadow and the non-conserved region is revealed by white background. **B**) and **C**) the corresponding phylogenetic trees of the PR55/B β and PR55/B δ (**C**) from four (**B**) or six (**C**) vertebrates. The phylogenetic tree for PR55/B β (**B**) was generated through comparative analysis of the coding sequences in human, mouse, frog and the present study using UPGMA calculation and the MEGA3.1 software. The phylogenetic tree for PR55/B δ (**C**) was generated using the same strategy and software through comparative analysis of the coding sequences from mouse, rat, chicken, frog, zebrafish and the present study.

reveal that PR55/B family members have important functions controlling animal development and maintaining adult tissue homeostasis of goldfish.

Contrast expression patterns of PR55/B β/δ are present in lower and higher vertebrate

The protein phosphatase-2A (PP-2A) is one of the major phosphatases in eukaryotes, and the holoenzyme

of PP-2A is a heterotrimer, which contains a scaffold A subunit, a catalytic C subunit and a regulatory B subunit.¹⁻⁷ Both A and C subunits exist in two isoforms which are encoded by different genes. In contrast, the B subunits exist in 26 or more isoforms and so far, four subfamilies of genes, PR55/B, PR61/B', PR72/B'', and PR93/PR110/B''' have been identified to code for these different isoforms.^{1,2,6,15,16} In the present studies, we have isolated two members of the PR55/B family from goldfish. Although one member of the goldfish

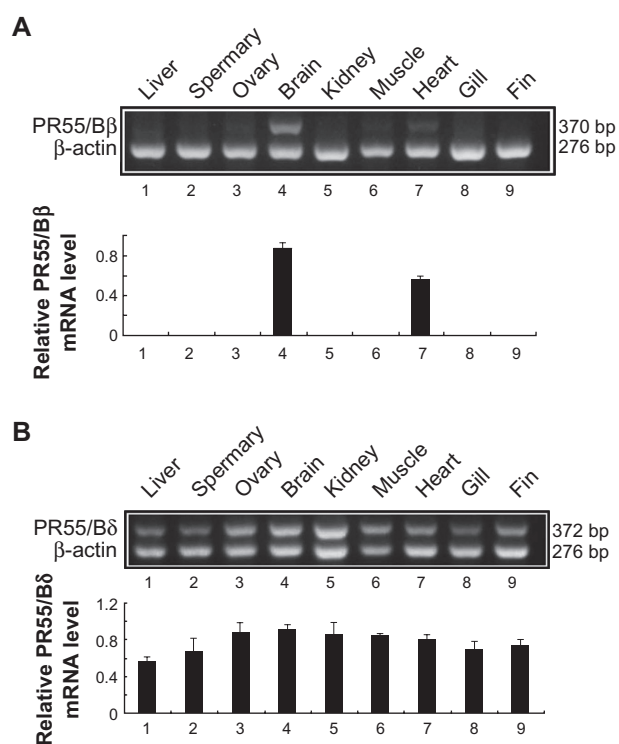


Figure 4. Tissue-specific expression of PR55/B β / δ mRNAs of PP-2A in adult goldfish. **A)** RT-PCR to detect the mRNA level of PR55/B β of PP-2A in 9 tissues of adult goldfish as indicated. Up panel: both the PR55/B β primers and the β -actin primers were added to the reaction at the beginning of PCR. The 370 bp DNA band for PR55/B β mRNA was detected in brain and heart. The 276 bp β -actin DNA band was detected in all tissues. Bottom panel: quantitative results of the PR55/B β mRNA levels from three independent experiments. The relative level of expression (fold) was calculated by dividing the total pixel from each PR55/B β mRNA band with the total pixel from the corresponding β -actin mRNA band. **B)** RT-PCR to detect the mRNA level of PR55/B δ of PP-2A in the same 9 tissues of adult goldfish as indicated in A. Like the 276 bp β -actin DNA band, the 372 bp DNA band for the PR55/B δ mRNA was also expressed in all the tissues examined. Bottom panel: quantitative results of the PR55/B δ mRNA expression from three independent experiments. The relative level of expression (fold) was calculated as described above in A.

PR55/B family shares high levels of amino acid identity (from 70% to 98%) with the counterpart from other vertebrates (Fig. 3), their expression patterns may be substantially different in different vertebrates. In the present study, we demonstrate that the goldfish PR55/B β mRNA is mainly expressed in the brain and to a much less degree in the heart. However, in mouse and rat, it is mainly expressed in testis and to a less degree in murine brain.^{27,28} For the expression pattern of PR55/B δ , there is significant difference between goldfish and murine. While in goldfish, PR55/B δ mRNA is expressed in all 8 tissues examined with the highest level found in brain, ovary and kidney, and the lowest level in liver and testis (spermary), the high level of PR55/B δ mRNA is only detected in mouse

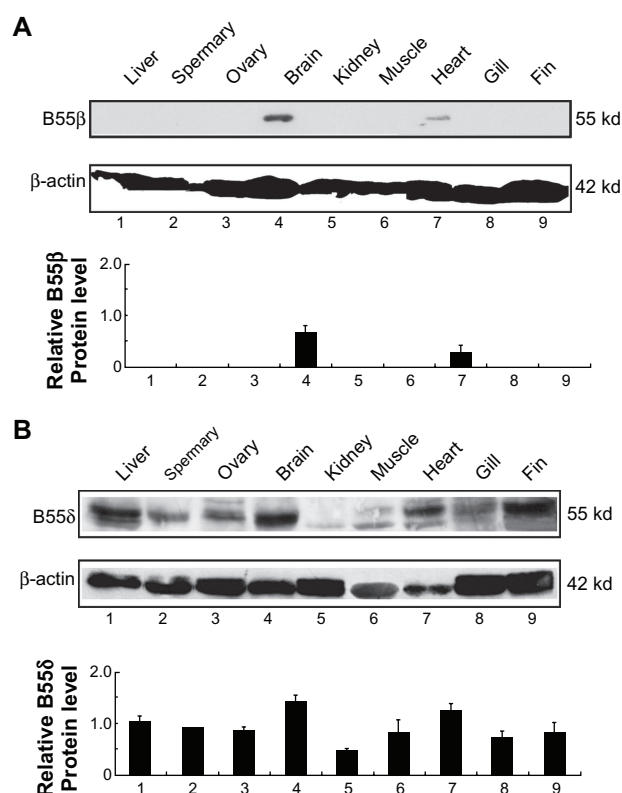


Figure 5. Western blot analysis of the PR55/B β and PR55/B δ proteins in 9 tissues of the adult goldfish indicated. **A)** Up panel: 100 μ g of total proteins extracted from the 9 different tissues of the adult goldfish were subjected to Western blot analysis as described in the Materials and Methods. Bottom panel: quantitative results of PR55/B β protein in the above 9 tissues of the adult goldfish from three independent experiments. Note that the highest expression levels of the PR55/B β protein was detected in the brain, and to a much less degree in the heart. **B)** Up panel: 100 μ g of total proteins extracted from the 9 different tissues of the adult goldfish were subjected Western blot analysis as described in A. Bottom panel: quantitative results of PR55/B δ protein in the above 9 tissues of the adult goldfish from three independent experiments. Note that the highest expression levels of the PR55/B δ protein was detected in the brain and heart, and a reduced level of this protein was detected in liver, spermary, ovary, gin and gill. A much reduced PR55/B β / δ protein expression was found in kidney.

testis, the remaining tissues either have very little PR55/B β mRNA (kidney, muscle, liver and brain) or no PR55/B δ mRNA (lung, spleen and heart).^{27–29} Thus, goldfish (lower vertebrate) and murine (higher vertebrate) display distinct difference in the tissue-specific expression patterns of PR55/B β / δ .

In the present study, we found that the PR55/B β mRNA was present at low levels at the first 3 stages and then became clearly upregulated at gastrulation stage. After a brief downregulation in neurula and optic vesicle stages, the PR55/B β mRNA were gradually increased from optic vesicle to heart beat stages and then maintained at this level with some slight fluctuations in the next three stages. Similar to

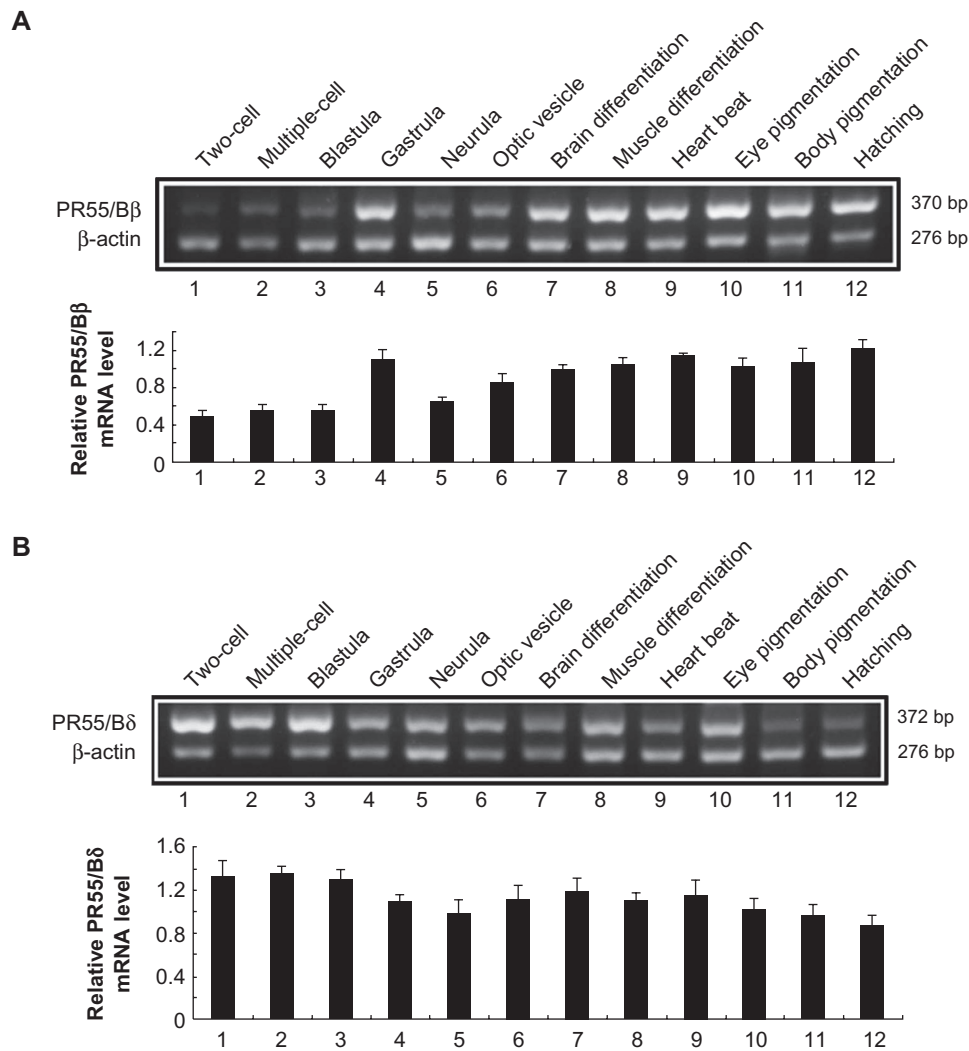


Figure 6. Temporal mRNA expression patterns of PR55/B β / δ for PP-2A during embryonic development of goldfish. **A)** Up panel: RT-PCR to detect the mRNA level of PR55/B β of PP-2A during 12 different developmental stages. Bottom panel: quantitative results of the PR55/B β mRNA expression from three independent experiments. **B)** Up panel: RT-PCR to detect the mRNA level of PR55/B δ of PP-2A during the 12 stages. Bottom panel: quantitative results of the PR55/B δ mRNA expression from three independent experiments. The method is the same as described in Figure 4.

the temporal mRNA expression pattern in goldfish, the PR55/B β mRNA was also detected in mouse embryo, as early as embryonic day 11 (ED11). This mRNA level became gradually increased from ED 11 to ED 17.²⁸ In contrast to the goldfish PR55/B β mRNA expression pattern, we hardly detected any PR55/B β protein expression at the 12 different developmental stages examined. Such results suggest that the PR55/B β mRNA may be non-translatable and the specific PP-2A activity with PR55/B β as regulatory subunit may be not necessary for goldfish development. Whether the PR55/B β mRNA in mouse embryo yields any detectable protein remains to be explored. On the other hand, we could not exclude the possibility that a low level of PR55/B β protein

exists that cannot be detected with the antibody we used and in the presence of a large portion of yolk protein in goldfish embryo. Different from the PR55/B β the PR55/B δ is highly expressed at both mRNA and protein levels from early to later developmental stages of goldfish. This temporal pattern is also different from that in mouse where no PR55/B δ transcripts could be detected until ED17.²⁸ Such distinct difference in their temporal expression patterns between lower and higher vertebrates suggest that PR55/B δ plays an important role in regulating development of goldfish embryo but not mouse embryo before ED17.

The discrepancy of the mRNA and protein levels for PR55/B δ in goldfish kidney (Figs. 5 and 6)

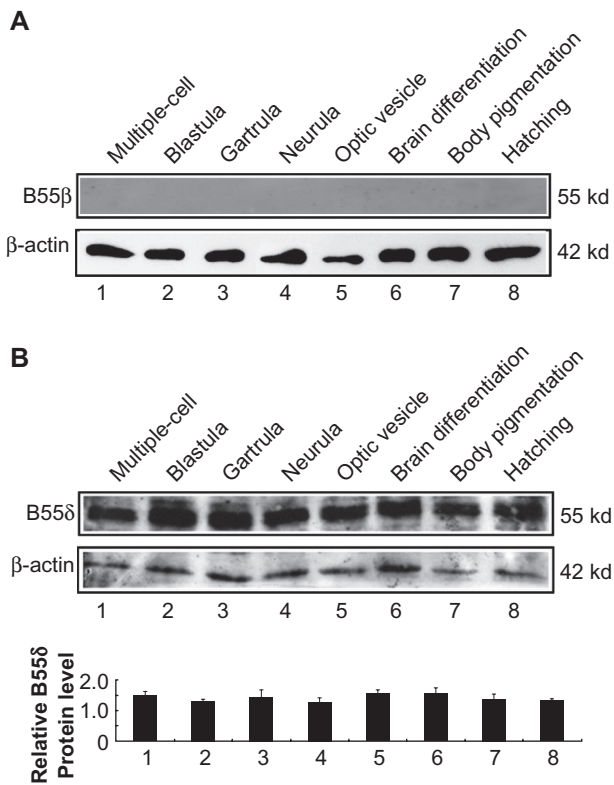


Figure 7. Western blot analysis of the protein for PR55/B β / δ in 8 developmental stages as indicated. **A)** Up panel: 100 micrograms of total proteins extracted from 8 developmental stages of the developing goldfish embryos were subjected to Western blot analysis. Note that no PR55/B β protein was detectable at any stage. **B)** Up panel: 100 micrograms of total proteins extracted from the 8 different stages were subjected to Western blot analysis as described in Figure 5. Bottom panel: quantitative results of PR55/B δ protein in the 8 developmental stages as determined using the methods described in Figure 5.

suggests that the other isoform of PP2A PP55/B family regulatory subunit, PR55/B α , may play an important role in regulating specific PP-2A activity in this tissue.

P55/B δ of PP-2A has important functions in regulating development

Besides its regulatory function in the heterotrimeric holoenzyme of PP-2A, the regulatory subunits of PP-2A may have other functions. In *Drosophila*, there exists only one form of the PR55/B regulatory subunit.³⁰ It has been shown that *Drosophila* mutants with reduced levels of PR55 expression display pleiotropic phenotypes.³¹ Although three mutant alleles, *aar*¹, *aar*² and *twins*^P, derived from the insertion of different P-elements at the same position within the PR55 gene all show mitotic abnormalities in anaphase, *aar*¹ displays abnormality in larval brain, *aar*² is female sterile, and *twins*^P shows imaginal disc abnormality.³²⁻³⁴ The imaginal disc duplication observed in *twins*^P is

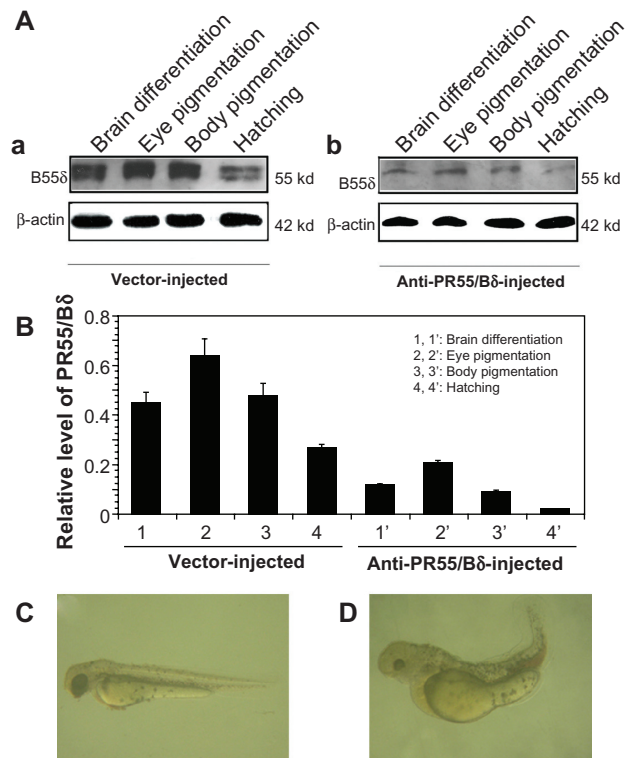


Figure 8. Inhibition of PR55/B δ protein expression through anti-sense blockage of translation led to severe phenotype in goldfish development. **A)** The relative expression levels of P55/B δ in vector-injected (**A-a**) and anti-sense P55/B δ expression construct-injected embryos (**A-b**). **B)** Quantitation of comparative expression in vector-injected and anti-sense P55/B δ expression construct-injected embryos. Note that anti-sense blockage of PR55/B δ translation led to a substantial decrease in the expression level of the PR55/B δ protein at 4 different stages. The down-regulation of the PR55/B δ protein caused severe abnormality of organogenesis in the eye and trunk (**D**) in comparison with the vector-injected embryos (**C**).

derived from complete loss of PR55/B expression.³⁴ These results indicate that the functions of PR55/B observed in *twins*^P can't be complemented by other PP-2A activity, suggesting presence of non-PP-2A functions of PR55/B.^{32,34} Since the three types of insertion mutations all affect the expression level of PR55/B in different tissues and the abnormality in the expression level of PR55/B leads to multiple phenotypes, it is conceivable that PR55/B has important functions in *Drosophila* development. In the present study, we found that PR55/B δ is highly expressed from two-cell stage to hatching larvae (Figs. 6 and 7). To explore its function in regulating development, we expressed the anti-sense RNA from the exogenous PR55/B δ cDNA, which can block the expressed PR55/B δ mRNA from the endogenous PR55/B δ gene. The principle of this technology is based on the fact that the anti-sense RNA can form complementary duplex with the sense mRNA strand and thus block the translation



of the later.³⁵ It has been extensively used for suppression of endogenous gene expression.^{36–38} Western blot analysis confirmed that expression of the anti-sense RNA substantially attenuated the protein expression level of PR55/B δ in goldfish embryos of different developmental stages (Fig. 8A & 8B). When PR55/B δ is downregulated, the development of goldfish embryos displays severe abnormality in organogenesis. We observed that during differentiation stage, while expression of the vector (mock) had little effect on the eye development, expression of the antisense PR55/B δ RNA led to microphthalmia and abnormal trunk in the embryos with reduced PR55/B δ expression of majority embryos (Table 2). These results provide the first evidence that the regulatory subunit of PP-2A directly controls eye and trunk development. Our demonstration that downregulation of PR55/B δ by anti-sense RNA led to microphthalmia (small eye) in the developing embryo suggests the specific PP-2A activity contributed by PR55/B δ regulatory subunit is crucial for development. In this case, the PP-2A containing PR55/B δ regulatory subunit may modulate a set of specific targets important for development that can't be dephosphorylated by PP-2A with non-PR55/B δ regulatory subunit. Indeed, previous studies have shown that such proteins as cdc25, histone H1 and caldesmon phosphorylated by p34^{cdc2}/cyclinB kinase are only subjected to dephosphorylation by the specific PP-2A containing PR55/B regulatory subunit.^{31,39–42} In addition, the PP-2A containing PR55/B regulatory subunit also regulates targets phosphorylated by MAP kinases such as the microtubule-associated protein, tau.^{43,44} On the other hand, we could not rule out the possibility that the PR55/B δ regulatory subunit alone functions in some unknown mechanism to govern goldfish development. Whether the later case is possible is currently under investigation.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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