

Expression patterns of SH3BGR family members in zebrafish development

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Abstract SH3 domain-binding glutamic acid-rich (SH3BGR) gene family is composed of *SH3BGR*, *SH3BGR1*, *SH3BGR2*, and *SH3BGR3* which encodes a cluster of small thioredoxin-like proteins and shares a Src homology 3 (SH3) domain. However, biological functions of SH3BGR family members are largely elusive. Given that zebrafish (*Danio rerio*) *sh3bgr1*, *sh3bgr2*, *sh3bgr3*, and *sh3bgr* are evolutionally identical to their corresponding human orthologues, we analyzed the spatiotemporal expression of *SH3BGR* family members in zebrafish embryonic development stages by in situ hybridization. Our results revealed that except *sh3bgr1*, other members are all maternally expressed, especially for *sh3bgr3* that is strongly expressed from one-cell stage to juvenile fishes. In situ expression patterns of *SH3BGR* members are similar in the very early developmental stages, including with commonly strong expression in intestines, olfactory bulbs, and neuromasts for neural system building up. Organ-specific expressions are also demonstrated, of which *sh3bgr* is uniquely expressed in sarcomere, and *sh3bgr3* in liver. *sh3bgr1* and *sh3bgr2* are similarly expressed

in intestines, notochords, and neuromasts after 12-h post-fertilization of embryos. Eventually, messenger RNAs (mRNAs) of all *sh3bgr* members are mainly constrained into intestines of juvenile fishes. Collectively, our study clarified the expression patterns of *sh3bgr* family members in diverse organogenesis in embryonic development and indicates that SH3BGR members may play predominant roles in neural system development and in maintenance of normal function of digestive organs, especially for intestine homeostasis. However, their expression patterns are varied with the development stages and organ types, suggesting that the aberrant expression of these members would result in multiple diseases.

Keywords SH3BGR family · Zebrafish · Development · Diseases

Introduction

SH3 domain-binding glutamic acid-rich-like (SH3BGR) was originally discovered in an attempt to find the causative genes of Down syndrome (Scartezzini et al. 1997) and is grouped into SH3BGR family with other three members (SH3BGR1, SH3BGR2, and SH3BGR3) (Mazzocco et al. 2001, 2002). These genes encode a cluster of small proteins with approximately 100 amino acids, which belongs to thioredoxin-like protein super family. Since then, SH3BGR and SH3BGR1 have been reported to be expressed in *Xenopus* (Jang et al. 2015), and all four members are also expressed in chicken (Majid et al. 2006). Structurally, the SH3BGR family proteins share a number of conserved domains, of which the proline-rich domain, Ean/Vasp homology 1 (EVH1)-binding domain, and Src homology 3 (SH3)-binding motif that have been linked to signal pathway transductions through protein-protein interactions (Egeo et al. 1998). Besides, the member

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SH3BGR contains an extra proline-rich and a glutamic acid-rich domain in the C-terminal region adjacent to the N-terminal thioredoxin-folding domain (Jang et al. 2015). Although the sh3bgr gene located in the critical region responsible for Down syndrome heart issue, *sh3bgr* overexpression did not result in the heart defect phenotype (Sandri et al. 2004). Recent study on Sh3bgr showed that it is critical for proper sarcomere formation in striated muscle tissues through regulating localization of Enah and heart development formation by affecting the Enah protein level in *Xenopus* (Jang et al. 2015). Downregulation of SH3BGRL plays a role in v-Rel-mediated transformation (Majid et al. 2006), while upregulation in triple-negative breast cancer (TNBC) (Muñiz Lino et al. 2014). Our recent results showed that SH3BGRL functions as a tumor suppressor in human, but its mutation can reverse it to a tumor promoter (Wang et al. 2015). The function of SH3BGRL2 is not well studied, except it is differentially expressed in lymph mononuclear cells between type 1 and type 2 diabetes (Collares et al. 2013). In spite of Sh3bgrl3 was recognized as a target of miR-1 and miR-206 in regulating C2C12 myoblast differentiation (Goljanek-Whysall et al. 2012), expression of Sh3bgrl3 and its possible functions during development is still insufficient. Likewise, there is no any detailed study on expression profile of *sh3bgr* family members in vertebrates' development, and it is thus necessary to shed light on possible developmental and physiological functions of sh3bgr family.

Given that zebrafish (*D. rerio*) is an excellent model to study both developmental role of a gene and its physiological relevance at whole body level, in this study, we analyzed the spatiotemporal expression of zebrafish *sh3bgr* family members identified previously as their human orthologues during embryonic development, in order to dissect their instinctive roles in pathogenesis upon aberrant expression during embryonic development.

Results and discussion

Comparison of Sh3bgr family protein sequence

Sh3bgr family proteins consist of Sh3bgr, Sh3bgrl, Sh3bgl2, and Sh3bgrl3 that belong to the SH3 domain-binding glutamic acid-rich proteins, but their gene loci are different. Zebrafish sh3bgr gene (NM_001001713) is located on chromosome 15 and encodes a 182 amino acid protein, while zebrafish sh3bgrl gene (NM_201209) on chromosome 14 with 115 amino acids encoded. Zebrafish sh3bgrl2 gene (NM_001002197) is located on chromosome 23 and encodes a 105 amino acid protein, and zebrafish sh3bgrl3 gene (NM_200278) on chromosome 19 with 92 amino acids. Each zebrafish SH3bgr member possesses a typical protein domain containing a thioredoxin-like fold, a SH3-binding domain, and a glutamate-rich domain. To

investigate the conservative level of the zebrafish sh3bgr family members, amino acid sequence alignment was performed. The alignment between each sh3bgr family member indicated a highly conserved SH3-binding domain involved (Fig. 1a), which is putatively involved in protein-protein interactions in signal transduction. Furthermore, to compare the evolutionary conservation of zebrafish sh3bgr family members to their corresponding human orthologues, we performed sequence alignments of human (Hs) and zebrafish (Dr) sh3bgr, sh3bgrl, sh3bgrl2, and sh3bgrl3, respectively. As shown in Fig. 1b, highly conservative sequences were observed between the individual human and zebrafish orthologues in terms of the amino acid sequences and the biochemical character of each amino acid., respectively. In addition, the binding sites of SH3 domain are also highly conserved (Fig. 1b).

Expression analysis of zebrafish sh3bgr family by RT-PCR

We sought to determine whether there are differences in expression patterns of the zebrafish sh3bgr family members. We used reverse transcription polymerase chain reaction (RT-PCR) to analyze the spatiotemporal expression patterns of sh3bgr family members during zebrafish embryonic development. Our results from Fig. 2 revealed that except sh3bgrl3 which is continuously highly expressed from one-cell stage to 96-h post-fertilization (hpf), the embryogenesis of zebrafish, all other members exhibited dynamic expression during the early stage of embryonic development, and the relative high levels were detected at 12 hpf onwards. sh3bgr and sh3bgrl2 expressions could be marginally detected at one-cell stage and increased with embryos development. In contrast, the detectable sh3bgrl expression was evidently delayed at 4 hpf, which is matched to the gastrula stage. Together, these results suggested that SH3BGR members could have their unique expression patterns during zebrafish development.

sh3bgr expression during zebrafish development by whole-mount in situ hybridization

To directly demonstrate the expression pattern of each sh3bgr member, we first used sh3bgr gene antisense RNAs labeled with digoxigenin to reveal the distribution of sh3bgr gene from one-cell stage to 96 hpf by the whole-mount in situ hybridization. Our results showed that sh3bgr is expressed in the apical part of zebrafish embryos as early as from one cell to the sphere stage (Fig. 3a, b). Up to 18 hpf, *sh3bgr* messenger RNA (mRNA) was concentrated in the neural tubes (Fig. 3c, d) and maintained at the central nervous system (CNS) until 24 hpf (Fig. 3e–g). Meanwhile, sh3bgr is also

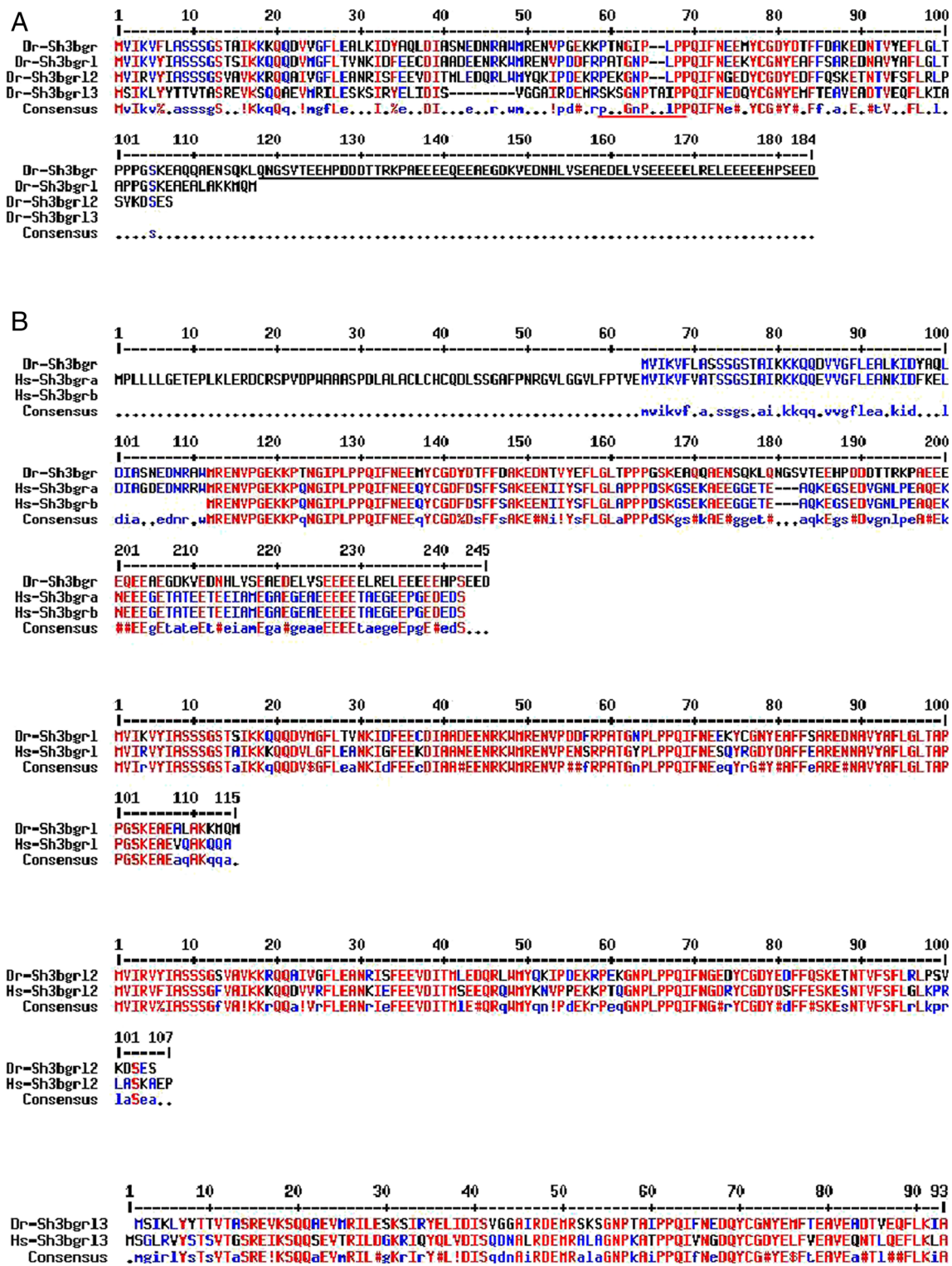


Fig. 1 Amino acid sequence alignments of zebrafish (Dr) sh3bgr family proteins. **a** Protein sequences of zebrafish sh3bgr family members show high conservation of SH3-binding regions that are *underlined in red*. In addition, SH3BGR protein contains an extra proline-rich and a glutamate-

rich domain *underlined in black*. **b** Protein sequence alignments between human and zebrafish SH3BGR members indicate the high conservation of sh3bgr family members in evolution

strongly expressed at myotome borders, consistent with the previously reported expression pattern of sh3bgr in *Xenopus*, suggesting that sh3bgr may play a role in the sarcomere

formation (Jang et al. 2015). At 48 hpf, *sh3bgr* expression still remained to be detected in sarcomere (Fig. 3h). From dorsal view, sh3bgr was expressed in the otic vesicle (Fig. 3i), and at

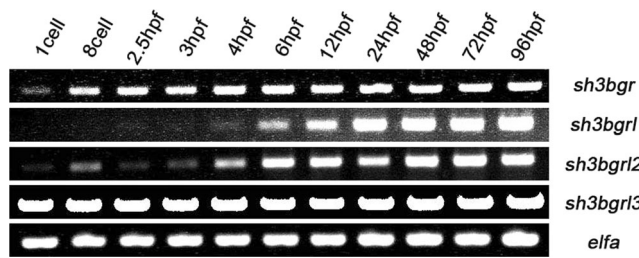


Fig. 2 RT-PCR analysis of *sh3bgr* family member's expression during zebrafish embryonic development. mRNAs of *Sh3bgr* family member were detected at indicated stage by RT-PCR. *Elfa* is served as a loading control

72 hpf, it was similarly observed in sarcomere (Fig. 3k). Transverse section also revealed *sh3bgr* expression in the sarcomere (Fig. 3l). From lateral view, *sh3bgr* expression was also clearly visible in neuromast, olfactory bulb, and pectoral fin buds from 72 h (Fig. 3m) to 96 hpf (Fig. 3n), and the only difference is that *sh3bgr* was faintly observed in intestines at 96 hpf (Fig. 3o). Till 120 hpf, *sh3bgr* was mainly restricted into the intestine (Fig. 3p). Taken together, we presented *sh3bgr* expression in developing zebrafish CNS, sarcomere, and intestine, suggesting that *sh3bgr* may have the potential roles on CNS formation and intestine function.

sh3bgr1 expression during zebrafish development

Likewise, we analyzed the expression profile of *sh3bgr1* during the embryonic development by whole-mount in situ hybridization. Unlike *sh3bgr* expression, no visible *sh3bgr1* signal was detected until the eight cell stage of zebrafish embryos (Fig. 4a), which is in line with the result of RT-PCR detection (Fig. 2), suggesting that *sh3bgr1* may be involved in later developmental machineries. Till 50 % of epiboly stage, a faint *sh3bgr1* signal was observed in epidermis (Fig. 4b), and about 12 hpf, we found *sh3bgr1* mRNA to be enriched in somites and tail buds (Fig. 4c). At 18 hpf, *sh3bgr1* was gradually concentrated in the CNS and neural tubes (Fig. 4d, e) and was maintained at the central nervous system until 24 hpf, and at the same time, we also detected *sh3bgr1* expression at otic vesicle (Fig. 4f, g). However, at 48 hpf, *sh3bgr1* transcripts were gradually restricted to some domains, and clearer signals were detected in notochord (Fig. 4h, i). In addition, we also found that *sh3bgr1* mRNA was presented in otic vesicles (Fig. 4j), which is very similar to the expression pattern of *sh3bgr* at 48 hpf. At 72 hpf embryo, the strongest expression appeared in neuromasts, olfactory bulbs, and pectoral fin buds (Fig. 4k, l). From 96 to 120 hpf, the majority of *sh3bgr1* mRNA is observed in the digestive tract, especially in intestines (Fig. 4m–o). Overall, *sh3bgr1* expression is

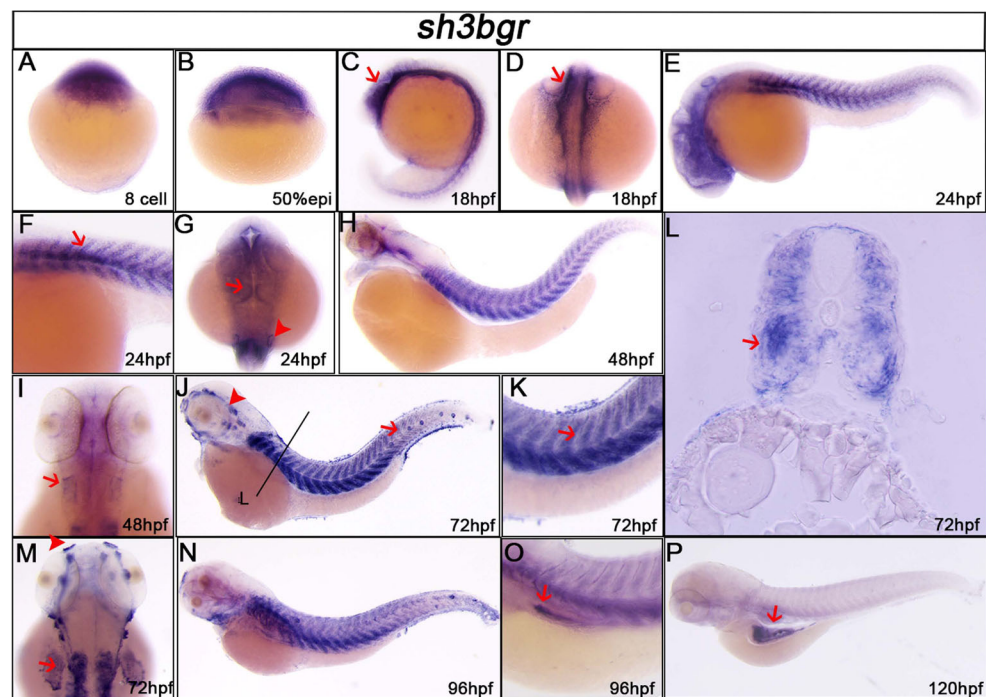


Fig. 3 Whole-mount in situ hybridization analyses of zebrafish *sh3bgr* in embryo development (eight cell–96 hpf) with its antisense probe. Stages at **a** Eight cell, lateral view with strong *sh3bgr* signal. **b** Fifty percent epiboly, lateral view. **c** 18 hpf, lateral view, the central neural system (CNS) (arrow). **d** 18 hpf, dorsal view, central. **e** 24 hpf, lateral view. **f** 24 hpf, lateral view, myotome borders (arrow). **g** 24 hpf, dorsal view CNS (arrow) and otic vesicle (arrowhead). **h** 48 hpf, lateral view. **i** 48 hpf,

dorsal view, otic vesicles (arrow). **j** 72 hpf, lateral view, anterior neuromasts (arrowhead), and posterior neuromasts (arrow). **k** 72 hpf, lateral view, myotome borders (arrow). **l** A transverse section showing *sh3bgr* expression. Arrow indicates the myotome borders. **m** 72 hpf, dorsal view, olfactory epithelium (arrowhead) and ventral fin (arrow). **n** 96 hpf, lateral view. **o** 96 hpf, lateral view, intestine (arrow). **p** 120 hpf, lateral view of intestine (arrow)

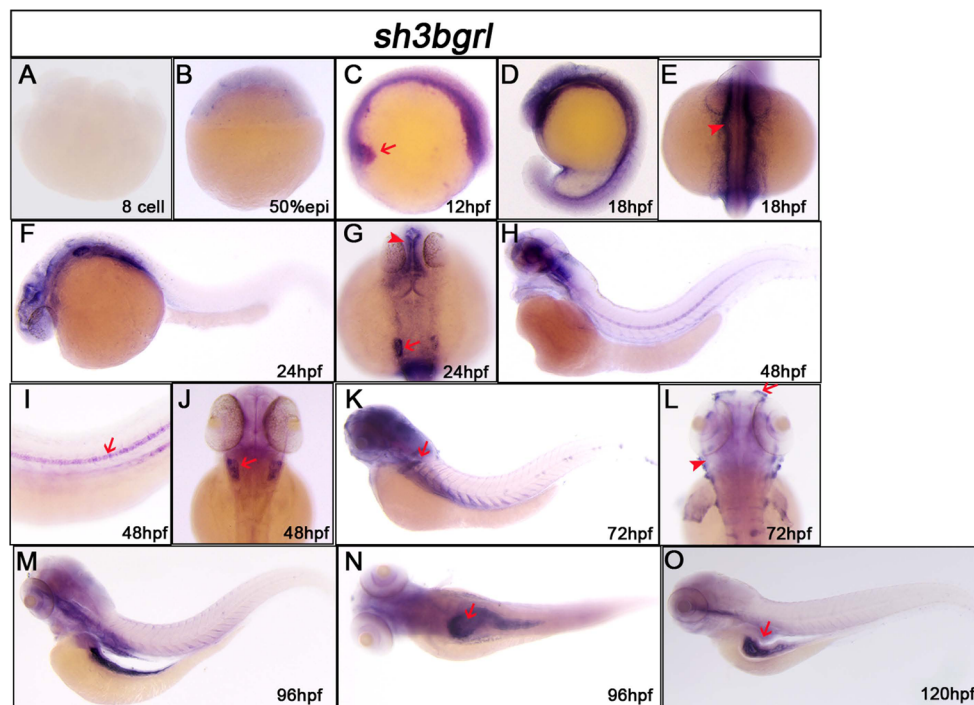


Fig. 4 Whole-mount in situ hybridization analyses of zebrafish *sh3bgr1* expression in embryo development using its antisense probe. Stages at **a** Eight cell, lateral view, no *sh3bgr1* hybridization signal. **b** Fifty percent epiboly, lateral view, weakly staining. **c** 12 hpf, lateral view, somite and tail bud (*arrow*). **d** 18 hpf, lateral view. **e** 18 hpf, dorsal view, central nervous system (*arrowhead*) and neural tube (*arrow*). **f** 24 hpf, lateral

view. **g** 24 hpf, dorsal view, CNS (*arrowhead*) and otic vesicles (*arrow*). **h** 48 hpf, lateral view. **i** 48 hpf, lateral view, notochord (*arrow*). **j** 48 hpf, dorsal view, otic vesicle (*arrow*). **k** 72 hpf, lateral view, ventral fin (*arrow*). **l** 72 hpf, dorsal view, olfactory epithelium (*arrow*) and neuromast (*arrowhead*). **m** 96 hpf, lateral view. **n** 96 hpf, dorsal view, intestine (*arrow*). **o** 120 hpf, lateral view, intestine is pointed with *arrow*

similar to *sh3bgr* expression in the term of nervous system formation. In contrast, zebrafish *sh3bgr1* was also detected in neuromasts, notochords, and intestines, indicating that the expression area of *sh3bgr1* is different than that of xenopus *sh3bgr1* (Jang et al. 2015). Here, we may conclude that *sh3bgr1* is mainly involved in CNS and intestine formation.

***sh3bgr2* expression during zebrafish development**

To determine spatiotemporal distribution of another member, *sh3bgr2* mRNA expression pattern, we carried out the similar in situ hybridization analyses with antisense *sh3bgr2*. Only at eight cell stage, *sh3bgr2* transcripts can be clearly detected (Fig. 5a), which was shown ubiquitous at 50 % epiboly stage (Fig. 5b). At somite stage of 18 hpf, *sh3bgr2* was obviously existed in the overall developing nervous system (Fig. 5c, d), and at 24 hpf, its expression was still detected in the central nervous system. Meanwhile, *sh3bgr2* began to appear in otic vesicles (Fig. 5e, f) and lasted up to 48 hpf (Fig. 5g, i). In addition, a strong signal of *sh3bgr2* was detected in the notochord at 48 hpf (Fig. 5g, h). Moreover, *sh3bgr2* was expressed in the anterior and posterior neuromasts, olfactory bulb until 96 hpf, as well as in pectoral fin bulbs at 72 hpf (Fig. 5j, k). At 96 hpf, *sh3bgr2* signal was gradually confined to the mouth (Fig. 5m) and intestinal bulbs (Fig. 5l). At 120 hpf, its expression is only

evidently detected in intestine (Fig. 5n). Taken together with the above all results, we predict that *sh3bgr2* might play a crucial role in CNS development and intestine formation.

***sh3bgr3* expression during zebrafish development**

Finally, we analyzed the expression of *sh3bgr3* with *sh3bgr3* antisense probes during the development of zebrafish. As shown in Fig. 6, *sh3bgr3* was originally and pervasively expressed in early developmental stages, including at the eight cell stage, implying its maternal gene feature (Fig. 6a, b). At 18 hpf, *sh3bgr3* expression was observed in the central nervous system, neural tubes, and the epidermis of the whole body (Fig. 6c, d). Around 24 hpf, *sh3bgr3* expression in the central nervous system and neural tube was still maintained (Fig. 6e–f), and the signal was also detected in otic vesicles (Fig. 6f) as well as in notochord (Fig. 6g). At 48 hpf, we found that *sh3bgr3* was expressed in notochord (Fig. 6h, j), as well as in the otic vesicles (Fig. 6i). Additionally, we can clearly detect that *sh3bgr3* is expressed in ciliary marginal zone (Fig. 6k). As a whole, this result is consistent with early study reported in ZFIN (direct submission by B. and C. Thisse). Moreover, our results distinctly indicated its detailed expression region in the eyes. From 72 to 96 hpf, a clear expression was observed in the anterior and posterior neuromasts (Fig. 6l, m), olfactory bulbs (Fig. 6m), and livers (Fig. 6l–n), as

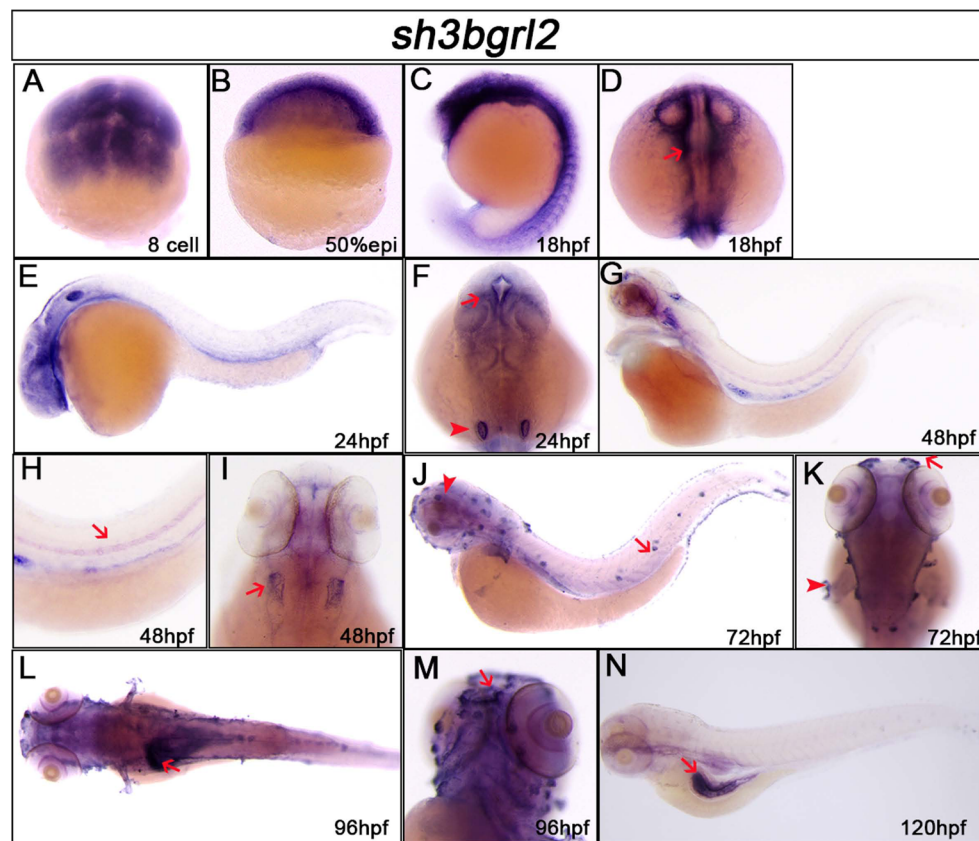


Fig. 5 Whole-mount in situ hybridization analyses of zebrafish *sh3bgrl2* during embryo development. Stages at **a** Eight cell, lateral view of the strong *sh3bgrl2* staining. **b** Fifty percent epiboly, lateral view. **c** 18 hpf, lateral view. **d** 18 hpf, dorsal view, central nervous system (*arrow*). **e** 24 hpf, lateral view. **f** 24 hpf, dorsal view, CNS (*arrow*) and otic vesicles (*arrowhead*). **g** 48 hpf, lateral view. **h** 48 hpf, lateral view,

notochord (*arrow*). **i** 48 hpf, dorsal view, otic vesicle (*arrow*). **j** 72 hpf, lateral view, anterior neuromasts (*arrowhead*) and posterior neuromasts (*arrow*). **k** 72 hpf, dorsal view, olfactory epithelium (*arrow*) and ventral fin (*arrowhead*). **l** 96 hpf, dorsal view, intestine (*arrow*). **m** 96 hpf, ventral view, mouth (*arrow*). **n** 120 hpf, lateral view of strong *sh3bgrl2* in intestine (*arrow*)

well as in the intestines (Fig. 6l–n). Transverse sections clearly manifested that *sh3bgrl3* is expressed in the intestine (Fig. 6o) and liver (Fig. 6p). In contrast to other three SH3BGR family members, *sh3bgrl3* was specifically expressed in the liver. Collectively, based on the above results, we concluded that *sh3bgrl3* may be participated in the formation of liver to maintain its function, besides in the early neural system development.

In summary, here we demonstrated that *sh3bgr* family members share a similar and overlapping expression pattern in various organs throughout early embryogenesis, suggesting that the aberrant expression of these members would result in multiple diseases. Meanwhile, our results also revealed that except *sh3bgrl*, other members are all maternally expressed, especially for *sh3bgrl3* that is strongly expressed from one-cell stage to all other developmental processes. The organ-specific expressions of the members are also observed at the early developmental stages and eventually all restricted to intestines, indicating that SH3BGR members may play predominant roles in neural system in early development and in maintenance of normal function of digestive organs, especially for intestine homeostasis.

Material and methods

Zebrafish (*D. rerio*) maintenance and embryo collection

Wild-type zebrafish and embryos were maintained at 28.5 °C in incubator. Embryos were obtained after the immediate mating between the wild-type fishes and incubated at 28.5 °C, which were fixed in terms of developing stage for gene expression analysis. All embryos at 24 hpf were decolorized by incubating in phenylthiourea (PTU) before being fixed.

RNA extraction, reverse transcription, and RT-PCR

Total RNA was isolated from embryos using TRIzol reagent (Ambion, USA) and stored at −80 °C, according to the manufacturer's protocol. Four micrograms of total RNAs was reverse-transcribed into complementary DNAs (cDNAs) with 1 μl of M-MLV reverse transcriptase (Promega, USA) in a final volume of 20 μl, following manufacturer's instructions. Synthesized cDNAs were stored at −20 °C. PCR amplification of each member's DNA fragments of SH3BGR family was performed in a

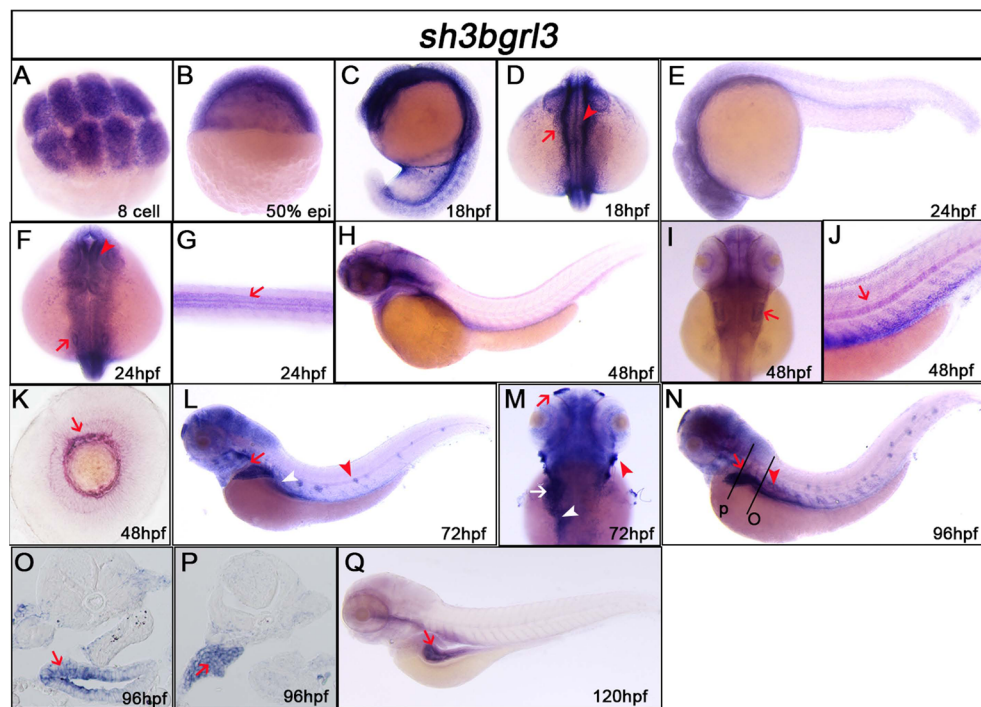


Fig. 6 Whole-mount in situ hybridization analyses of zebrafish *sh3bgrl3* in embryo development. Stages at **a** Eight cell, lateral view with strong *sh3bgrl3* staining. **b** Fifty percent epiboly, lateral view. **c** 18 hpf, lateral view. **d** 18 hpf, dorsal view, central nervous system (*arrow*) and neural tube. **e** 24 hpf, lateral view. **f** 24 hpf, dorsal view, CNS (*arrowhead*) and otic vesicles (*arrow*). **g** 24 hpf, lateral view, notochord (*arrow*). **h** 48 hpf, lateral view. **i** 48 hpf, dorsal view, otic vesicle (*arrow*). **j** 48 hpf, lateral view, notochord (*arrow*). **k** A magnified view of the eye at 48 hpf; *arrow* indicates ciliary marginal zone. **l** 72 hpf, lateral view, posterior

neurocasts (*arrowhead*), liver (*arrow*), and intestine (*white arrowhead*). **m** 72 hpf, dorsal view, anterior neurocasts (*red arrowhead*), olfactory epithelium (*red arrow*), liver (*white arrow*), and intestine (*white arrowhead*). **n** 96 hpf, lateral view, liver (*arrow*) and intestine (*arrowhead*). **o**, **p** Transverse sections showing *sh3bgrl3* expression in intestine (**o**) and liver (**p**). *Arrow* and *arrowhead* indicate intestine and liver, respectively. **q** 120 hpf, lateral view of *sh3bgrl3* expression in intestine (*arrow*)

final volume of 25 μ l, with their corresponding primers at a final concentration of 0.5 μ M each and 0.13 μ l of GoTaq DNA polymerase (Promega, Madison, WI, USA). The primers are listed in Table 1, including primer pairs of *sh3bgrF/sh3bgrR*, *sh3bgrIF/sh3bgrIR*, *sh3bgrl2F/sh3bgrl2R*, and *sh3bgrl3F/sh3bgrl3R*, in which F indicates the forward primer, and R the reverse direction. The PCR parameters are 2 min for template annealing at

95 $^{\circ}$ C; 35 cycles of amplification at 95 $^{\circ}$ C for 50s, 58 $^{\circ}$ C for 50s, and 72 $^{\circ}$ C for 45 s, and with a final extension at 72 $^{\circ}$ C for 5 min. A fragment of *elfa* was used as a control whose primers were *elfaF* and *elfaR* (Table 1), and amplification is similar to that for SH3BGR members, except the annealing temperature is 60 $^{\circ}$ C. Amplified PCR products were run on a 1.0 % TAE/agarose gel and visualized by ethidium bromide staining.

Table 1 Primer sequences used in this study

Name	Sequence (5'-3')	Application
<i>elfaF</i>	CTGATCTACAAATGCGGTGGA	For RT-PCR
<i>elfaR</i>	GCAGGGTTGTAGCCGATCTTC	For RT-PCR
<i>sh3bgrF</i>	ACCTTCACCGCAGCTTTACCC	For probe and RT-PCR
<i>sh3bgrR</i>	CTAGA GCCCGCCTTACGCAT	For probe and RT-PCR
<i>sh3bgrIF</i>	GGAAA CCCCTTACCA CCTC	For probe and RT-PCR
<i>sh3bgrIR</i>	AGCGGCTAATAATTTGACTT	For probe and RT-PCR
<i>sh3bgrl2F</i>	GAAGCCCTCACTGCCAGCGAT	For probe and RT-PCR
<i>sh3bgrl2R</i>	TGTCCAAATGTAGAGAGCCTG	For probe and RT-PCR
<i>sh3bgrl3F</i>	ATCCTCTCCCTTCACTGGT C	For probe and RT-PCR
<i>sh3bgrl3R</i>	ACAGAAATCTATTGGCACGGT	For probe and RT-PCR

Generation of sh3bgr1, sh3bgr2, sh3bgr3, and sh3bgr probes

To avoid cross-hybridization between sh3bgr1, sh3bgr2, sh3bgr3, and sh3bgr, the 3' UTR regions of sh3bgr1, sh3bgr2, sh3bgr3, and sh3bgr were clone into the PGEM-T easy vector (Promega). All sequences were amplified by RT-PCR as above PCR procedures. For synthesis of DIG-labeled antisense RNA probes, the plasmids of sh3bgr1, sh3bgr2, sh3bgr3, and sh3bgr were linearized with Pst1 and transcribed with T7 RNA polymerases according to the kit recommends of the DIG RNA label kit (Roche).

Whole-mount in situ hybridization and imaging

For whole-mount in situ hybridization, embryos were incubated at 28 °C, then fixed at the appropriate developing stages in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C overnight, and washed with PBS containing 0.1 % Tween 20 (PBST) four times, 5 min each. Embryos after 24 hpf were empirically treated with protease K (1:1000 dilution), followed by washing with PBST four times, 5 min each again. The fixed embryos were pre-hybridized buffer (50 % formamide, 5 × SSC, 0.1 % Tween, 50 μg/μl tRNA, pH 6.0 with citric acid) for at least 2 h at 65 °C. Subsequently, the embryos were incubated overnight at 65 °C in the same buffer containing digoxigenin-labeled RNA probes at 1.5 ng/μl of final concentration and incubated overnight at 65 °C, followed by first washing at 65 °C for 20 min with the washing buffer (50 % SSC, 50 % formamide), second for 20 min in 75 % SSC, 25 % formamide, third for 20 min in 2 × SSC and two times in 0.2 × SSC, 15 min each, then followed with washing two times, 10 min each at room temperature with the MAB buffer (0.1 M NaCl, 0.1 % Tween, 0.15 M maleic acid pH 7.5) with gentle shaking, and then in blocking buffer for 1 to 3 h at room temperature. The embryos were further incubated with anti-digoxigenin antibody (1:3500 in dilution) in blocking buffer overnight at 4 °C and washed four times with PBST as above at room temperature. The embryos were subsequently rinsed two times, 10 min each in staining buffer (0.1 M NaCl, 0.05 M MgCl₂, 0.1 M Tris-HCl pH 9.5, 0.1 % Tween), and stained by NBT and BCIP dissolved in the same buffer to develop the optimal signals. The embryos can be kept in 4 % PFA at 4 °C. The stained embryos were mounted in 60 % glycerol and observed under a Leica M205FA, and then photos were taken with Leica DFC550.

Frozen section of the whole-mount in situ hybrid embryos and imaging

The whole-mount in situ hybridized embryos were permeabilized with 30 % of sucrose and embedded with cryo-

embedding media (OCT). Embedded embryos were slowly submerged into liquid nitrogen to ensure completely frozen and subsequently transferred to a cryotome cryostat at −20 °C prior to sectioning. The frozen tissues were sliced into 10-μm-thick sections using cryotome (Leica CM 1900). The sections were put on slides and covered for photograph as described in the “Whole-mount in situ hybridization and imaging” section.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicting interests.

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