ZOOLOGICAL RESEARCH

Expression analysis of eight amphioxus genes involved in the Wnt/β-catenin signaling pathway

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ABSTRACT

The Wnt/β-catenin signaling pathway plays a crucial role in the embryonic development of metazoans. Although the pathway has been studied extensively in many model animals, its function in amphioxus, the most primitive chordate, remains largely uncharacterized. To obtain basic data for functional analysis, we identified and isolated seven genes (*Lrp5/6*, *Dvl*, *APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro*) of the Wnt/β-catenin signaling pathway from the amphioxus (*Branchiostoma floridae*) genome. Phylogenetic analysis revealed that amphioxus had fewer members of each gene family than that found in vertebrates. Whole-mount *in situ* hybridization showed that the genes were maternally expressed and broadly distributed throughout the whole embryo at the cleavage and blastula stages. Among them, *Dvl* was expressed asymmetrically towards the animal pole, while the others were evenly distributed in all blastomeres. At the mid-gastrula stage, the genes were specifically expressed in the primitive endomesoderm, but displayed different patterns. When the embryo developed into the neurula stage, the gene expressions were mainly detected in either paraxial somites or the tail bud. With the development of the embryo, the expression levels further decreased gradually and remained only in some pharyngeal regions or the tail bud at the larva stage. Our results suggest that the Wnt/β-catenin pathway might be involved in amphioxus somite formation and posterior growth, but not in endomesoderm specification.

Keywords: Wnt/β-catenin signaling pathway; Gene expression; Amphioxus; Whole-mount *in situ* hybridization; Embryo

INTRODUCTION

The Wnt/β-catenin signaling pathway acts as a major route passing signals from the outside to the inside of a cell. The signaling is initiated by binding of Wnt ligands to Frizzled receptors and Lrp5/6 co-receptors, which in turn results in recruitment of Axin and Dvl to the cell membrane and succedent disassembly of β-catenin from the Apc-Axin-Gsk3β-CkI complex. After that, the released β-catenin proteins translocate to the nucleus and interact with the Tcf/Lef transcription factor to activate target gene expression (Logan & Nusse, 2004; Rao & Kuhl, 2008). Comparison among available genome sequences shows that members of this pathway are conserved throughout all metazoan clades, but not among fungi, plants, or unicellular eukaryotes (Holstein, 2012).

The conservation of the Wnt/β-catenin signaling pathway indicates its important role in metazoic embryogenesis. Indeed, functional studies have demonstrated that the signaling controls many aspects of metazoic development, including germ layer specification, axis patterning, and posterior growth (Hikasa & Sokol, 2013; Martin & Kimelman, 2009; Petersen & Reddien, 2009). In vertebrates, the signaling plays an early role in dorsal-ventral (D-V) axis determination and a late role in anterior-posterior (A-P) axis development and posterior growth regulation (Hikasa & Sokol, 2013; Martin & Kimelman, 2009). In invertebrate deuterostomes, such as *Ciona*, sea urchins, and hemichordates, the signaling is essential for both endomesoderm specification and A-P axis development (Darras et al., 2011; Imai et al., 2000; Logan et al., 1999; McCauley et al., 2015; Momose et al., 2008; Momose & Houliston, 2007; Wikramanayake et al., 1998, 2003). In protostomes, the function of this signaling pathway exhibits considerable divergence among taxonomic groups. For example, in *Caenorhabditis elegans* and *Drosophila melanogaster*, the

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signaling pathway regulates cell asymmetric division (Mizumoto & Sawa, 2007), but in nemertean *Cerebratulus lacteus* it participates in endoderm and A-P axis formation (Henry et al., 2008) and in short-germ band insects and spiders, it plays an essential role in posterior growth.

It appears that the function of the Wnt/β-catenin signaling pathway evolved distinctively during metazoan evolution, but questions about this evolvement have not yet been fully addressed. To increase our understanding of this pathway, we analyzed the expression profiles of eight genes involved in Wnt signaling at various developmental stages in amphioxus embryos. Amphioxus occupies a key position in the phylogeny of chordates (Delsuc et al., 2006), and its embryogenesis shows similarities to that of invertebrates (before the gastrula stage) and vertebrates (after the neurula stage). Importantly, amphioxus embryos have a clear A-P axis (Holland & Holland, 2007) and a posterior growth zone (tail bud) (Schubert et al., 2001), making them ideal research models to clarify the above questions. Previous studies have indicated that Wnt signaling is likely involved in amphioxus A-P axis determination, but not in D-V specification (Holland et al., 2005; Onai et al., 2009, 2012). However, whether signaling regulates amphioxus endomesoderm specification and posterior growth still remains to be elucidated.

MATERIALS AND METHODS

Animals and embryos

Originally provided from Jr-Kai Yu's lab by Dr. Zhang in 2011, amphioxus *Branchiostoma floridae* was introduced to our lab and cultured as described previously for *B. belcheri* (Li et al., 2012, 2013). Mature males and females with well-developed gonads were induced to spawn via thermal shock (Li et al., 2013). The eggs were fertilized *in vitro* and raised in dishes. Embryos at different developmental stages, including one-cell, two-cell, four-cell, blastula, mid-gastrula, early neurula (neural plate), mid-neurula (5-6 somites), late neurula (10-15 somites), and early larva (mouth open), were collected and fixed in 4% paraformaldehyde (PFA) dissolved in 4-morpholinepropanesulfonic acid (MOPS) buffer for whole-mount *in situ* hybridization (WISH).

Gene identification and cDNA cloning

Sequences of amphioxus *Lrp5/6*, *Dvl*, *APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro* genes were retrieved from the NCBI database using the Tblastn program with mouse homologous sequences as queries, and further verified with our transcriptome dataset. Primer pairs for each target gene were then designed (Table 1) and used to amplify the gene fragments using cDNA templates derived from gastrulae and neurulae. The amplified fragments were separately purified using a gel extraction kit (Omega, USA), subcloned into pGEM-T easy vector (Promega, USA), and verified by sequencing analysis.

Phylogenetic analysis

For each target gene, homologous sequences from *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Ciona intestinalis*, *Saccoglossus kowalevskii*, and *Strongylocentrotus purpuratus* were downloaded from public databases and aligned using the ClustalW program in MEGA5 software (Tamura et al., 2011). The alignment files were then used to construct neighbor-joining (NJ) trees using MEGA5 software with the Poisson model and 500 bootstrap replications.

Table 1 Primer sequences used for cDNA cloning and vector-construction

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Lrp5/6	GACCGATGCCTAGCCAAGT	CTTCTCGCCAGCAGGAGGATT
Dvl	ATGGAGGAGACGAAAATCATT	TCACATGACGTCCACGAAG
Axin	ATGAGTATTGGTGTACAGGAATAC[0]	TCACTCTATGCGCTCCACCTT
Apc	AGCTCGGTTGCTACAGGAGATAG	GTGCCGACAAGTTCCACAAA
CkIα	GGTACCCTTTGGGACAGAATGGCGAGC	ACTAGTCACCTCAGCGTCCTTTAC
CkIδ	AGAGCCGAGATGGAGTTGA	CTCCGACTACTTCTTGATG
Gsk3β	ATGAGCGGAAGACCACGGACAAC	TTATGTCCCCCCAGAGGCTGATGCG
Gro	ATGTATCCTCAAAACAGAC	TCAGTAGATGACTTCGTAAAG

Whole-mount *in situ* hybridization

Digoxigenin-labeled (Roche, USA) sense and antisense probes for target genes were synthesized using Sp6 or T7 RNA polymerase from the templates of linearized recombination pGEM-T easy vectors, and WISH was performed according to previously reported methods (Yu & Holland, 2009), with slight modification. Before the WISH experiments, envelopes of unhatched embryos were removed with a slim glass pin to facilitate reagent penetration. After proper staining, the embryos were fully washed with PBS, and were then mounted in 80% glycerol and photographed under an inverted microscope.

RESULTS

Identification of Wnt/β-catenin signaling components in amphioxus

Several genes encoding the components in the Wnt/β-catenin signaling pathway have been described in amphioxus in previous research (Holland, 2002; Lin et al., 2006; Qian et al., 2013; Yu et al., 2007). In the present study, we isolated another seven genes, including *Lrp5/6*, *Dvl, APC*, *CkIα*, *CkIδ*, *Gsk3β*, and *Gro*, from the genome of *Branchiostoma floridae*. Results

indicated that amphioxus possesses all genes involved in the Wnt/β-catenin signaling pathway, but fewer homologous genes than that observed in vertebrates (Supplementary Figure 1-7 and Supplementary Table 1). For example, two Wnt co-receptor genes (*Lrp5* and *Lrp6*) exist in the vertebrate genome, but a single ortholog (*Lrp5/6*) was found in the amphioxus genome; three *Dvl* genes exist in the genomes of most vertebrate species, but only one counterpart was detected in amphioxus. These observations are consistent with the hypothesis of a pre-duplicated genome status in amphioxus, indicating that components of the Wnt/β-catenin signaling pathway have not experienced lineage-specific expansion and should be much simpler than that in vertebrates.

Expression pattern of genes involved in the Wnt/β-catenin signaling pathway

To investigate the function of the Wnt/β-catenin signaling pathway during amphioxus embryogenesis, we further determined the expression patterns of the seven newly identified genes using the WISH method. The *Axin* gene was included in the present analysis since its expression at early development stages has not been determined in previous studies (Beaster-Jones et al., 2008). Figures 1, 2, and 3 are overviews of the gene expression patterns. From the one-cell to late blastula stages, the transcripts of *Lrp5/6*, *Axin*, *APC*, *Ckl*α, *Ckl*δ, *Gsk3β*, and *Gro* appeared evenly throughout the developing embryos, but that of *Dvl* was asymmetrically localized towards the animal pole (Figure 1, column A to D), which was denoted by the *Vasa* gene expression at the opposite vegetal pole (Figure 1A2, B2). At the gastrula stage, the expressions of the genes became undetectable in the ectoderm and conspicuously appeared in the primitive endomesoderm (Figure 1, column E and F). Interestingly, these genes were differentially expressed in the endomesoderm, with *Lrp5/6* mainly expressed in the dorsal and lateral regions, *Dvl* and *Gro* primarily expressed in the dorsal-anterior region, *APC* and two *Ckl*s expressed in the dorsal region, *Axin* expressed in the blastopore rim, and *Gsk3β* evenly expressed throughout the endomesoderm. These patterns were typically maintained until the early neurula stage, except that the signal in the dorsal mesoderm became more conspicuous than that in other tissue (Figure 2, column A to C). At the mid and late neurula stages, the expressions of these genes were commonly detected in the paraxial mesoderm (somites) (Figure 2, column D and E; Figure 3, column A and B). Other expression sites were also detected for the *Axin* gene in the tail bud, *Dvl* and *Gro* in the anterior endoderm diverticulum, and *Gsk3β* in the gut. At the larva stage, the expressions of the genes decreased and weak signals remained in some pharyngeal regions or the tail bud only (Figure 3, column C). We also synthesized sense probes of *Gsk3β*, *Gro*, and *APC* genes and performed side-by-side *in situ* hybridization experiments using both sense and antisense probes. No signal was observed in the embryos hybridized with the sense probes, but a strong specific staining was observed in the embryos hybridized with the antisense probes (Supplementary Figure 8).

DISCUSSION

Function of the Wnt/β-catenin signaling pathway in amphioxus early embryogenesis

The activation of Wnt/β-catenin signaling on one side of a cleavage-stage embryo is a crucial process in metazoan embryonic axis patterning and germ layer specification. In animals such as sea urchins (Logan et al., 1999), hemichordates (Darras et al., 2011), and *Ciona* (Imai et al., 2000), early regionalized Wnt signaling is localized to the vegetal pole and associated with endomesoderm specification. In cnidarians, the signaling is involved in endomesoderm specification (Martindale, 2005), although it is activated in the animal pole (Wikramanayake et al., 2003). In vertebrates, however, the signaling activity is enriched in the dorsal region, where it is crucial for Spemann-Mangold organizer formation and subsequent D-V axis determination (Hikasa & Sokol, 2013; Niehrs, 2004). This early regionalized Wnt signaling center is generally activated by localized expression of upstream signaling components such as Wnt ligands (Tao et al., 2005) and/or Dvl (Miller et al., 1999; Weitzel et al., 2004; Tadjuidje et al., 2011). To date, ten Wnt genes have been identified in amphioxus and eight of their developmental expression patterns have been analyzed by *in situ* hybridization; however, none of these genes have shown a detectable expression before the gastrula stage (Holland, 2002). Using RT-qPCR, five *Wnt* genes, including *Wnt1*, *6*, *9*, *10*, and *11*, were found to be maternally expressed, although expression levels were relatively low (Qian et al., 2013). However, it is not clear whether these genes are expressed asymmetrically at one pole of cleavage-stage embryos. In the present study, the amphioxus *Dvl* gene was asymmetrically expressed toward the animal pole in the amphioxus embryo from the one-cell to late blastula stages. This expression pattern is the same as that of amphibian *Dvl* (Miller et al., 1999; Tadjuidje et al., 2011), but different from that of sea urchin *Dvl*, which is enriched in the vegetal pole (Weitzel et al., 2004). Thus, it appears unlikely that amphioxus embryos could form a regionalized Wnt signaling center at the early stages via the asymmetric expression of the *Dvl* gene since the downstream effector β-catenin can localize nuclei of all embryonic blastomeres from the 16-cell to late blastula stages (Holland et al., 2005). This possibility is further strengthened by the ubiquitous expression of *Lrp5/6*, *Axin*, *APC*, *Ckl*α, *Ckl*δ, *Gsk3β*, and *Gro* (present study) and four *Frizzled* genes (Qian et al., 2013), and the undetectable expression of Wnt/β-catenin signaling antagonists such as *Dkk*s, *sFrp*s, and *Ceberus* in amphioxus embryos before the gastrula stage (Yu et al., 2007; Onai et al., 2010, 2012; unpublished data). Results suggest that Wnt signaling is probably not involved in endomesoderm specification or D-V axis determination in amphioxus (Holland et al., 2005). Consistent with this assumption, the upregulation of Wnt signaling through the inhibition of Gsk3β activity by lithium chloride before the mid-blastula stage has no effect on mesendoderm specification or D-V axis development (Holland et al., 2005). The evidence supporting amphioxus having a regionalized Wnt/β-catenin signaling center in the cleavage-stage embryo is the

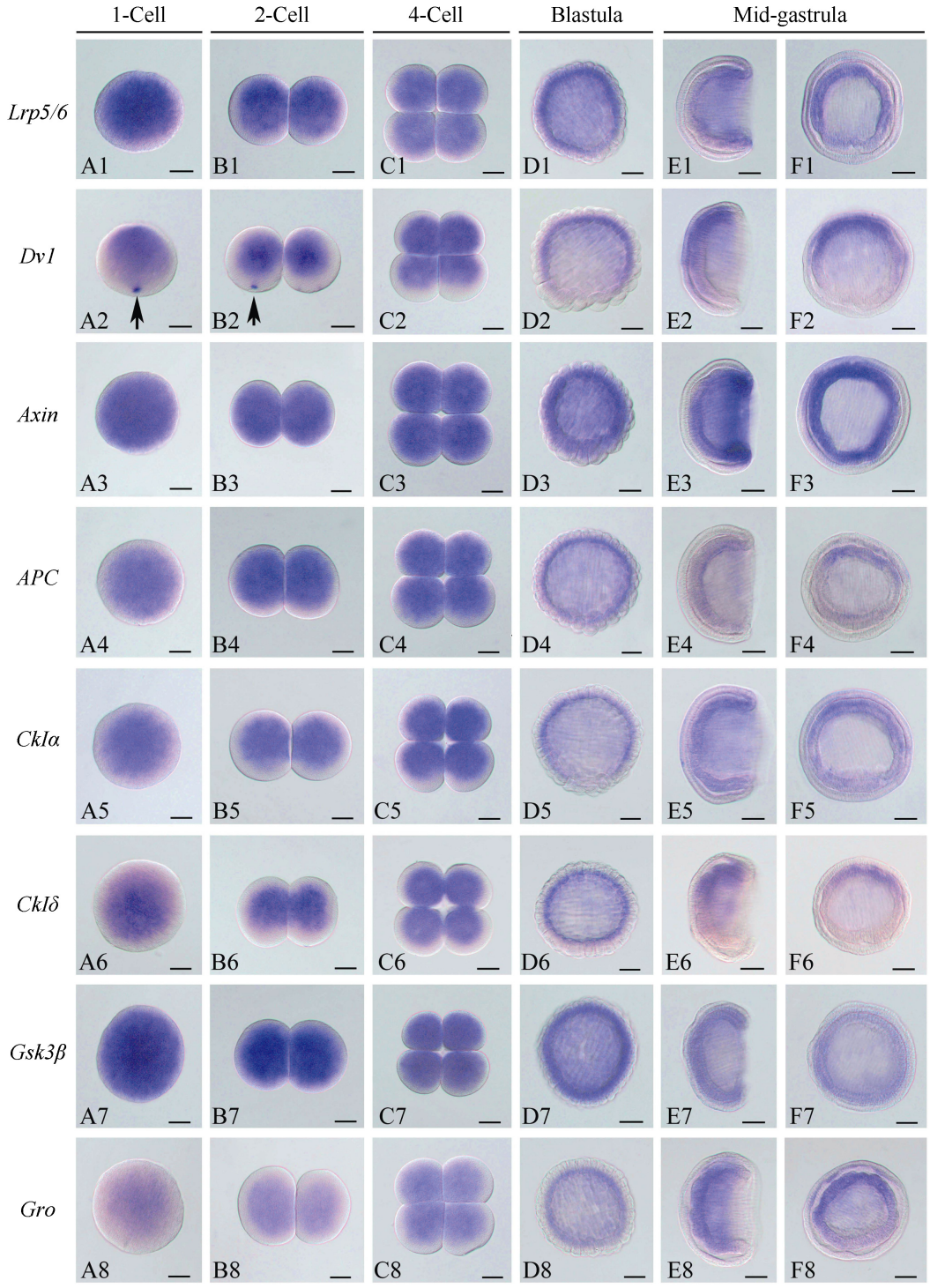

Figure 1 Overview of the expression patterns of eight Wnt/β-catenin signaling genes in one-cell to mid-gastrula stages of amphioxus embryos
Embryos in columns A, B, D, and E are in side view, embryos in column C are in animal-vegetal axis view, and those in column F are in blastopore view. Embryos in A2 and B2 were hybridized with *Dvl* and *Vasa* (vegetal marker) probes simultaneously to show the asymmetrical expression of *Dvl* in the animal pole. Scale bars=50 μm.

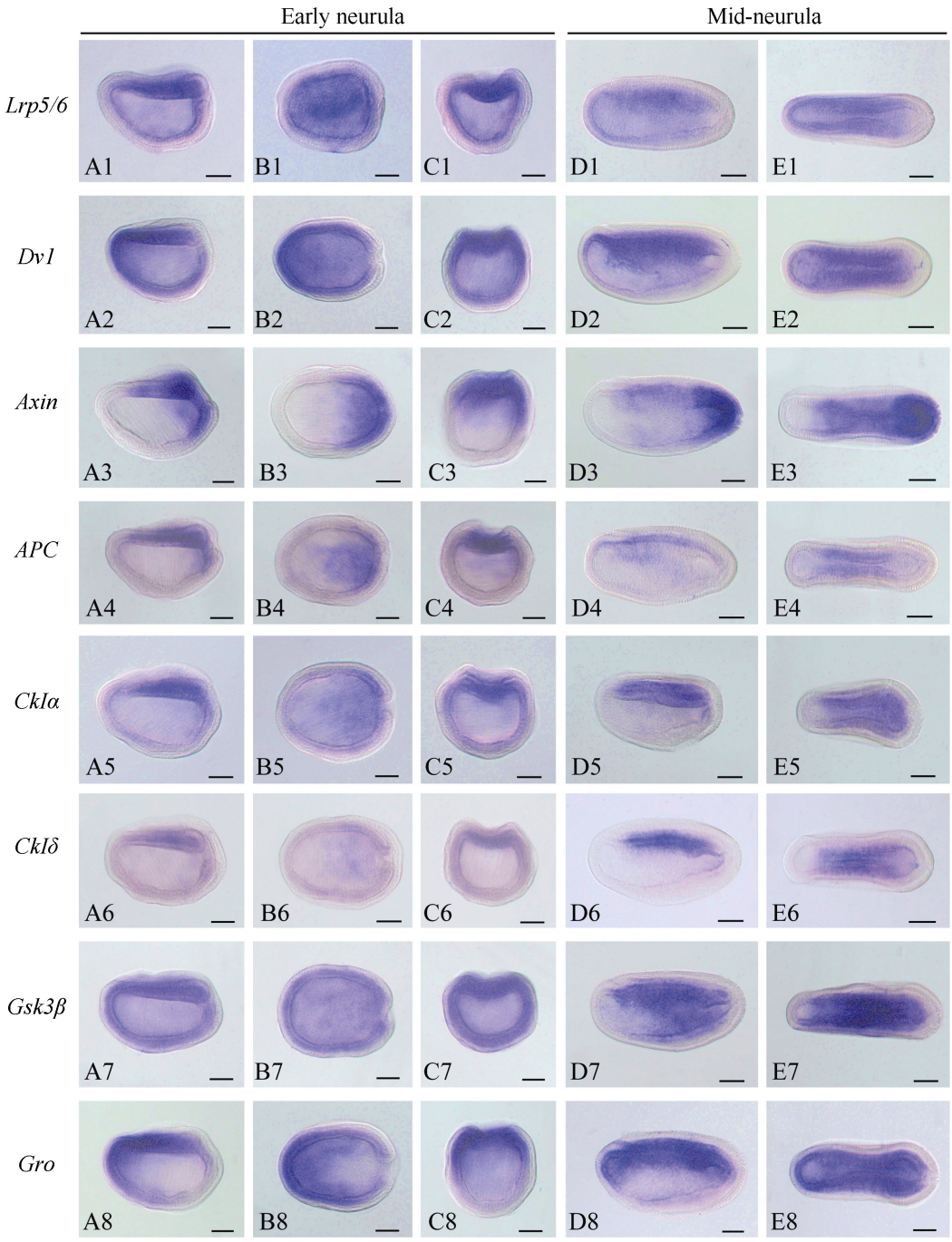

Figure 2 Overview of the expression patterns of eight Wnt/β-catenin signaling genes in early and mid-neurula stages of amphioxus embryos

Embryos in columns A and D are in side view, embryos in column C are in blastopore view, and those in columns B and E are in dorsal view. Scale bars=50 μm.

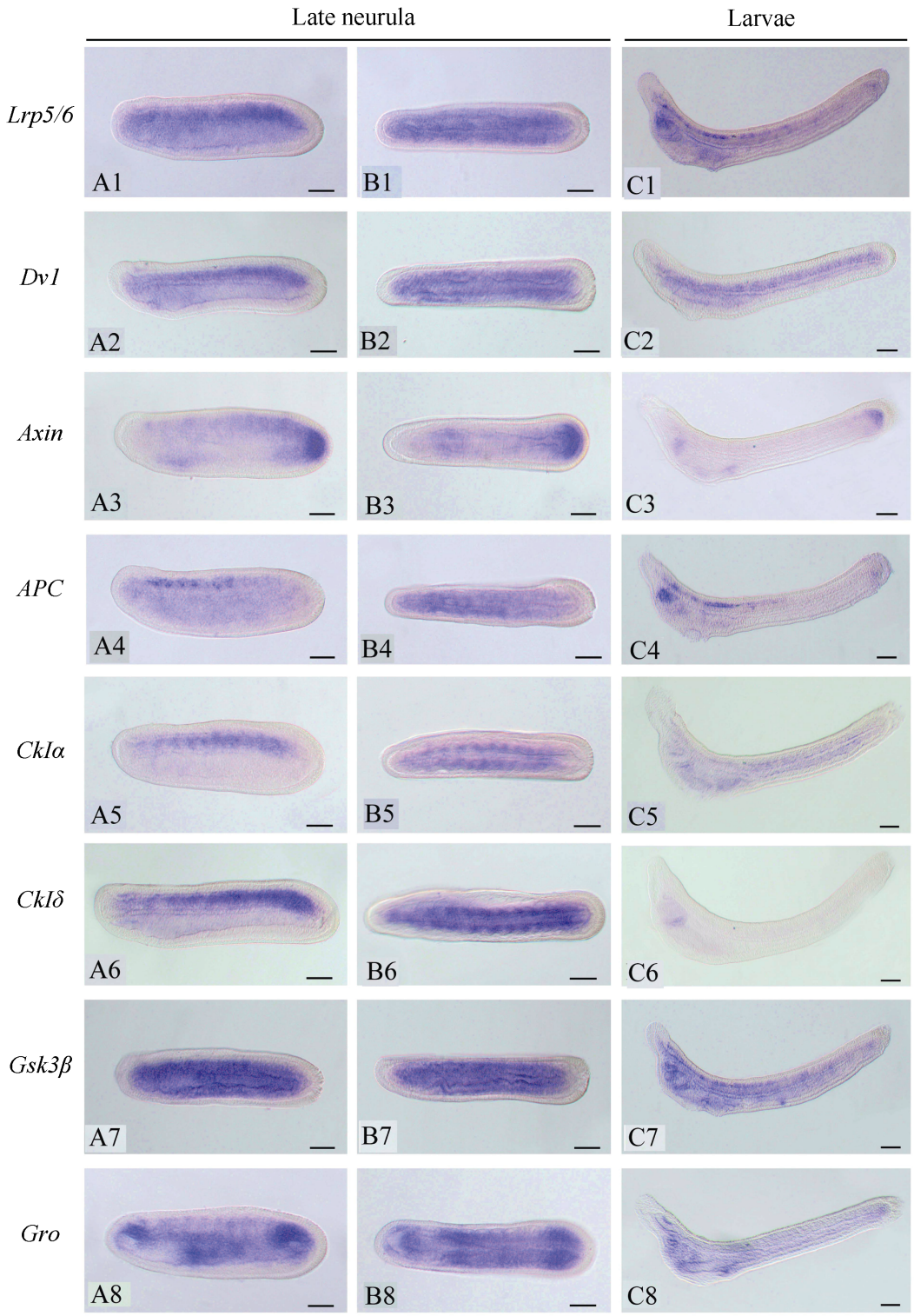

Figure 3 Overview of the expression patterns of eight Wnt/β-catenin signaling genes at late neurula and early larva stages
Figures in columns A and C are in side view and those in column B are in dorsal view. Scale bars=50 μm.

asymmetrical expression of the *Tcf* gene in the animal pole of early stage embryos (Lin et al., 2006). It would be interesting to perform functional study on the amphioxus *Tcf* gene via over-expression and knockdown experiments in the future.

Function of Wnt/β-catenin signaling in amphioxus posterior growth and somitogenesis

Body elongation via posterior growth is an important feature during the late stage of vertebrate embryogenesis. This elongation is accomplished by means of continuously adding segmented somite blocks to the posterior region (tail bud) at the neurula stage. Wnt signaling plays an essential role during the process by coordinating mesoderm formation and segmentation (Dunty et al., 2008). Vertebrate embryos lacking Wnt signaling components like Wnt3a or β-catenin display posterior axis truncations and form only head structures and anterior parts of the trunk (Dunty et al., 2008; Takada et al., 1994). Posterior growth does not exist in laboratory non-invertebrate metazoan animals such as sea urchins, *Ciona*, *Drosophila*, and *Caenorhabditis elegans*. This makes determining whether Wnt signaling regulates posterior growth in animals outside of vertebrates elusive. However, several studies on short germ-band insects, including the red flour beetle and cricket, have indicated a conserved role for Wnt signaling in posterior growth (Bolognesi et al., 2008; Miyawaki et al., 2004), although the genetic network underlying it is relatively different between insects and vertebrates (Martin & Kimelman, 2009). Amphioxus embryos have a posterior growth zone (tail bud) from which posterior somites can continuously bud off in a one-left-one-right manner (Schubert et al., 2001). Based on the expressions of *Wnt* and target genes, such as *Brachyury* and *Caudal*, within or around the tail bud of amphioxus embryos, Holland (2002) speculated that the Wnt signaling pathway should play a critical role in amphioxus posterior growth. Studies on other amphioxus Wnt signaling components, including *β-catenin* (Holland et al., 2005), *Tcf* (Lin et al., 2006), *Frizzled*s (Qian et al., 2013), *Lrp5/6*, *Dvl*, *Axin*, *Ck*s, *Gsk3β*, and *Gro* (present study), have further strengthened this speculation as these genes were transcribed within or around the amphioxus tail bud region. These results have also implicated that posterior growth mediated by Wnt/β-catenin signaling might be an ancient characteristic in metazoans and the genetic toolkit underlying this process would be relatively complete in amphioxus.

The Wnt/β-catenin signaling pathway is also essential for early stages of somitogenesis in vertebrates by maintaining the expression of myogenesis gene *MyoD* and inducing axial mesoderm inhibitor genes *Vent*/*Vox* (Hoppler & Moon, 1998; Ramel & Lekven, 2004). However, after the commitment of paraxial mesoderm to somatic fate, Wnt signaling is rapidly downregulated and not required for late myogenesis (Tian et al., 1999). Preliminary analysis on *Wnt8* expression patterns indicated a similar role for Wnt signaling in amphioxus early somitogenesis (Schubert et al., 2000). It also revealed that amphioxus *Wnt8* was continuously transcribed in some of the differentiated somites, indicating an essential role for Wnt/β-catenin signaling in both early stage somite formation and somite differentiation (Schubert et al., 2000). Consistent with this observation, we found that amphioxus *Lrp5/6*, *Dvl*, *Axin*, *APC*, *Ck*s *Gsk3β*, and *Gro* were all expressed in most differentiated somites in the mid to late neurulae. Further functional experiments are necessary to clarify this hypothesis.

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