Published in final edited form as:
Dev Dyn. 2009 July ; 238(7): 1788–1795. doi:10.1002/dvdy.21977.

Zebrafish *wnt3* is Expressed in Developing Neural Tissue

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Abstract

Wnt signaling regulates embryonic patterning and controls stem cell homeostasis, while aberrant Wnt activity is associated with disease. One Wnt family member, Wnt3, is required in mouse for specification of mesoderm, and later regulates neural patterning, apical ectodermal ridge formation, and hair growth. We have identified and performed preliminary characterization of the zebrafish *wnt3* gene. *wnt3* is expressed in the developing tailbud and neural tissue including the *zona limitans intrathalamica* (ZLI), optic tectum, midbrain-hindbrain boundary, and dorsal hindbrain and spinal cord. Expression in these regions suggests that Wnt3 participates in processes such as forebrain compartmentalization and regulation of tectal wiring topography by retinal ganglia axons. Surprisingly, *wnt3* expression is not detectable during mesoderm specification, making it unlikely that Wnt3 regulates this process in zebrafish. This lack of early expression should make it possible to study later Wnt3-regulated patterning events, such as neural patterning, by knockdown studies in zebrafish.

Keywords

zebrafish; wnt3; wnt3l; wnt3a; neuron; neuronal; nervous system; CNS; neural patterning; ZLI; zona limitans; tectum; Wnt pathway; Wnt signaling; canonical Wnt signaling

INTRODUCTION

The Wnt signaling pathway regulates many events in embryonic development and is pivotal in controlling adult stem cell homeostasis. Dysregulation of Wnt signaling has been linked to numerous cancers and other diseases (Logan and Nusse, 2004; Clevers, 2006; Klaus and Birchmeier, 2008). Wnt genes have been highly conserved throughout evolution and mammals have genes for nineteen Wnt ligands. A recent census identified putative orthologues for many of these genes in chicken, frog, and fish (Garriock et al., 2007). Study of these genes, because of their pivotal role in development, stem cell biology, and disease, has been intense.

Although Wnt ligands have historically been classified as either “non-canonical” or “canonical” ligands, some recent results have suggested that the distinction between these pathways may be more fluid than previously thought, with particular Wnts able to activate both canonical and non-canonical pathways depending on the particular configuration of receptors present (Tao et al., 2005; Bovolenta et al., 2006; Mikels and Nusse, 2006; Hendrickx and Leyns, 2008; Nusse, 2008). A number of non-canonical pathways are beginning to be defined, but many of the specifics remain to be elucidated (Veeman et al., 2003; Semenov et al., 2007). Canonical signaling proceeds by activation of the transcription of target genes through the stabilization of the multi-functional protein, β-catenin. Under

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non-signaling conditions, cytosolic and nuclear levels of the signaling pool of β-catenin are kept low by the actions of a constitutively active phosphorylation complex known as the "destruction" complex, which includes the scaffolding protein, Axin, the protein encoded by the *adenomatous polyposis coli* locus (APC), and Glycogen Synthase Kinase-3 (GSK3), as well as ancillary proteins. Ligand binding to co-receptors of the Frizzled and LDL-receptor related protein (LRP) families leads to disruption of the destruction complex and stabilization of β-catenin, which translocates to the nucleus where it can bind DNA-binding proteins of the Tcf/Lef family and activate transcription of Wnt-responsive target genes (Logan and Nusse, 2004).

In anamniotes, the earliest patterning event controlled by the Wnt signaling pathway is specification of the dorsal axis. Stabilization of β-catenin on the future dorsal side of the embryo leads to activation of dorsal organizer genes such as *chordin* (*chd*), and eventually formation of head and dorsal structures (Schier and Talbot, 2005). Following the onset of zygotic transcription, Wnt factors such as Wnt8 and Wnt3a then ventralize the developing body plan (Schier and Talbot, 2005).

In mouse, Wnt3 plays one of the earliest roles in embryonic patterning among Wnt ligands. Targeted deletion has revealed that murine *Wnt3* is required for primitive streak formation and specification of embryonic mesoderm (Liu et al., 1999; Barrow et al., 2007). *Wnt3* transcripts are first detected in the proximal epiblast at embryonic day 6.0 (E6.0) and soon after are found throughout the growing primitive streak (Liu et al., 1999). Wnt3-null animals fail to generate mesoderm or definitive endoderm, although anterior-posterior patterning of visceral endoderm remains intact. Thus, Wnt3 is required for formation of embryonic mesoderm in mice (Liu et al., 1999).

A number of later developmental processes are also regulated by Wnt3, such as hair growth (Millar et al., 1999; Kishimoto et al., 2000), apical ectodermal ridge (AER) formation (Barrow et al., 2003), and neural patterning (Krylova et al., 2002; Braun et al., 2003; Lie et al., 2005; Schmitt et al., 2006; Lewis et al., 2008). In humans, a nonsense mutation in *WNT3* causes the rare genetic disorder Tetra-amelia syndrome, which results in limbless development and other abnormalities (Niemann et al., 2004).

Neural patterning in vertebrates is extensively regulated by Wnt signaling (Wilson and Houart, 2004; Ille and Sommer, 2005; Malaterre et al., 2007), and Wnt3 participates in this process. *Wnt3* is expressed in the prospective dorsal thalamus up to the border of the *zona limitans intrathalamica* (ZLI), throughout the superior colliculus (optic tectum in chick), and in the developing hindbrain and dorsal spinal cord (Roelink et al., 1990; Roelink and Nusse, 1991; Salinas and Nusse, 1992; Bulfone et al., 1993; Parr et al., 1993; Braun et al., 2003; Robertson et al., 2004). In mouse and chick, Wnt3 patterns the forebrain by helping to delimit the anterior and posterior forebrain (Braun et al., 2003; Lewis et al., 2008). Wnt3 regulates retinal ganglion axon guidance and the topography of tectal wiring through a repulsive interaction with the Ryk receptor, which has recently been shown to have high affinity for Wnts (Schmitt et al., 2006). Finally, Wnt3 likely regulates synapse formation between motoneurons and sensory neurons in the spinal cord (Krylova et al., 2002). Unfortunately, loss of function studies in mouse to elucidate the neural patterning functions of Wnt3 have been impaired by the fact that Wnt3 is required at earlier stages for primitive streak formation and specification of mesoderm (Liu et al., 1999; Lewis et al., 2008), although conditional deletion strategies have been helpful in revealing some later functions such as regulation of AER formation (Barrow et al., 2003).

Despite the importance of Wnt3 in vertebrate body plan formation, cloning and characterization of a zebrafish *Wnt3* orthologue has not yet been reported, although a

putative *wnt3* locus in the genome has been identified (Garriock et al., 2007). Here we report the cloning and preliminary characterization of zebrafish *wnt3*.

RESULTS

Discovery of the zebrafish *wnt3* gene

To determine if there is a zebrafish gene homologous to mouse *Wnt3*, we searched for genes that had been annotated as *wnt3* in the zebrafish genomic database at Ensembl.org (http://www.ensembl.org), and found EntrezGene LOC569420 that corresponded to the predicted reference sequence XM_692803. This locus was also described in a recent census of vertebrate *Wnt* genes, but the message was not identified (Garriock et al., 2007). We designed PCR primers to amplify the entire putative coding sequence from cDNA, but we were unable to amplify a product. One possible explanation for our inability to amplify the putative sequence was that the 5'-most coding exon given in Ensembl could be incorrectly predicted, thus there would be no transcripts containing the 5' primer sequence. We compared the mouse transcript to the putative zebrafish transcript and noticed that the homology deteriorated upstream of the most 5' splice junction in the open reading frame, also suggesting that the 5'-most exon was incorrectly annotated. We therefore queried the translated peptide corresponding to the mouse 5'-most coding exon against the zebrafish Ensembl genome by TBLASTN, which yielded a single hit, directly upstream of the putative *wnt3* coding exons. These observations suggested that the predicted transcript is incorrect and that the true transcript contains sequence from a different 5'-exon. In support of this hypothesis, two ESTs were found that contained the putative upstream exonic sequence. A nucleotide sequence closely conforming to our new prediction was recently deposited in GenBank under accession number EU203154.

We successfully amplified a zebrafish *Wnt3* homologue by designing a new 5' primer corresponding to a portion of the 5' untranslated region (5'-UTR) using the alternative upstream exonic sequence. The amplified cDNA contained 1131 base pairs (bps) including 1068 bps of putative open reading frame (ORF), with an in-frame stop codon upstream of the putative initiation codon in the 5-'UTR. The protein encoded by this ORF is 90% identical and 97% similar to the mouse and human Wnt3 proteins (Fig. 1 A). By comparison, the known zebrafish Wnt3l protein (also known as Wnt3a) is 82% identical and 90% similar to the mouse and human Wnt3 proteins. Phylogenetic analysis shows that our novel protein sequence segregates most closely with human and mouse Wnt3, while the known Wnt3l segregates with mouse Wnt3a (Fig. 1 B). A comparison of the loci of *Wnt3* in human and mouse to that of our newly discovered gene on chromosome 12 shows that synteny is conserved, with the identified zebrafish gene having the same 3'-proximal neighbor, *nsfb*, as in mouse (*Nsf*) and human (*NSF*; Fig. 1 C; (Garriock et al., 2007)). Synteny with the 5'-neighbor is not conserved in either of the two fish genomes examined. Based on the high identity between mouse and human Wnt3 and our novel gene, as well as conservation of synteny, we propose that our novel zebrafish gene be named *wnt3*. Zebrafish *wnt3* has been deposited in GenBank under the accession number XXXXX, and the corresponding protein sequence XXXXX.

Zebrafish *wnt3* is expressed in the developing nervous system

Because Wnt3 has been shown to have important roles during embryonic development in other organisms, we wanted to determine where and when zebrafish *wnt3* is expressed.

We first examined the course of temporal expression in whole embryos by reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 2 A). RNA was isolated from embryos at different time points during development, and the presence of *wnt3* transcripts

was assayed. We found that *wnt3* is first expressed at tailbud stage (10 hours post fertilization; hpf) and levels increased through 50 hpf, the latest time point examined. Notably, *wnt3* transcripts are not detected at shield or earlier (Fig. 2 A). By whole mount in situ hybridization (WISH), expression continues through at least 6 days post fertilization (dpf, Fig. 2 B u). These results indicate that, unlike in mouse, zebrafish *wnt3* is not expressed prior to the specification of the germ layers and is thus unlikely to play a role in primary axis specification or mesoderm induction. We cannot exclude the possibility that Wnt3 protein is maternally deposited. *wnt3* is expressed, however, over the time points relevant to neural and mesodermal patterning.

To determine where *wnt3* is expressed during embryogenesis, we performed WISH at various time points during development (Fig. 2 B). *wnt3* expression is first observed weakly in the presumptive forebrain, with notable expression at the site of the future ZLI (Fig. 2 B a, black arrowhead) and in the developing tailbud, especially at the lateral boundaries of the embryonic tailbud (Fig. 2 B d). Even at this early time point, expression is clearly different from that of *wnt3l*, which is expressed more strongly in the future tectum (Fig. 2 B b, c) and in the interior tailbud (Fig. 2 B e, f). At 18-somites (18-s., 18 hpf), expression of *wnt3* remains in the tailbud, but as at earlier times, is much more superficial than *wnt3l* (Fig. 2 B g–i). By 22-s. (20 hpf), *wnt3*, but not *wnt3l*, is expressed in short, bilateral stripes (Fig. 2 B j, k, red arrowheads, and data not shown) just medial to the finbud progenitor fields marked by *fgf24* (Fig. 2 B j, l, black arrowheads) (Draper et al., 2003). These bilateral stripes of expression are already apparent at 18-s. and disappear soon after 24 hpf (data not shown). Expression is not observed in the finbuds at later timepoints (e.g. Fig. 2 B t).

During somitogenesis, *wnt3* remains expressed in the developing nervous system and is readily detectable in the presumptive ZLI, diencephalon, the dorsal mesencephalon, the midbrain-hindbrain boundary (MHB) and progressively less strongly along the dorsal hindbrain and neural tube (Fig. 2 B m, p). Striations are visible in the hindbrain (Fig. 2 B m), and expression is weakly detectable in the basal plate of the midbrain (Fig. 2 B p) at 18-s. This expression overlaps that of *wnt3l*, with some significant differences (Fig. 2 B m–r). *wnt3* is expressed much farther into the ventral midbrain. In addition, *wnt3*, unlike *wnt3l*, is expressed in the prospective cerebellum (Fig. 2 B p, q). By 24 hpf (Fig. 2 B r), expression remains in all of the regions previously observed, and is now stronger in the ventral midbrain, where basal plate expression overlaps the ventral boundary of *sonic hedgehog a* (*shha*) expression (Fig. 2 B r). Expression is also strong in the dorsoanterior dienchepalon extending deeply into the ZLI. This expression is maintained at 30 hpf (Fig. 2 B s). At 4 and 6 dpf (Fig. 2 B t, u), a graded pattern of expression remains in the optic tectum with strongest expression in the dorsomedial tectum and at the rostral and caudal borders. Expression is also observable in the otic vesicles, but not in the posterior portion of the embryo (not shown). Sense controls showed no signal (not shown).

At all times examined, *wnt3* expression is overlapping to, but distinct from that of *wnt3l* (Fig. 2 B a–i, m–q). In particular, *wnt3l* has broader and stronger expression in the tailbud (Fig. 2 B d–i), while *wnt3* shows much stronger expression in the presumptive cerebellum and presumptive ZLI (Fig. 2 B m–q). In addition, *wnt3* is expressed in the basal plate from 17–18-somites to 30 hpf (Fig. 2 B p, r, s) making it unique among the known canonical zebrafish diencephalic and mesencephalic *wnt* genes: *wnt1*, *wnt3l*, *wnt7*, *wnt7b*, *wnt8b*, and *wnt10b* (Krauss et al., 1992; Macdonald et al., 1994; Lekven et al., 2001; Lekven et al., 2003; Buckles et al., 2004).

Zebrafish Wnt3 can activate the canonical Wnt pathway

The canonical Wnt pathway centers on β-catenin/Tcf-mediated transcriptional activation of target genes. We wanted to determine whether Wnt3 is able to activate the canonical

pathway. To assay canonical Wnt activity by Wnt3, we examined transcriptional activation of canonical Wnt targets in response to injection of *wnt3* mRNA. We injected embryos heterozygous for a Wnt-responsive transgene, in which *gfp* expression is driven by a minimal promoter with multiple Lef/Tcf binding sites upstream (*Tg*(*TOP:GFP*)^{w25}) (Dorsky et al., 2002). As a second readout, we examined transcription of the Wnt target *chd*, which is involved in dorsal axis specification and shows expanded embryonic expression in the presence of ectopic canonical Wnt activity (Moon et al., 1993; Du et al., 1995; Schier et al., 1996; Ober et al., 2006).

To determine whether Wnt3 can activate the canonical Wnt pathway, we injected single cell embryos from a TOP:GFP heterozygous outcross, and examined the effects on *gfp* and *chd* mRNA expression by WISH (Fig. 3). Although TOP:GFP transgenics do have Gfp protein that is observable by fluorescence microscopy, the transgene is expressed weakly, and fluorescence is not observable until later in development (Dorsky et al., 2002), we therefore used WISH as an earlier and more direct readout of transcriptional activation. Both *gfp* (Fig. 3, top row) and *chd* (Fig. 3, bottom row) show a dose-dependent increase in transcription of these canonical Wnt target genes. As expected, 50% of injected embryos assayed for *gfp* did not carry the transgene (since they were the progeny of a heterozygous outcross) and therefore did not show any *gfp* transcript, serving as an internal control (data not shown). These results indicate that Wnt3 is capable of activating the canonical Wnt pathway.

DISCUSSION

We have identified a previously uncharacterized *wnt3*-like gene in the zebrafish genome. Based on the fact that the deduced Wnt3 protein segregates most closely with human and mouse Wnt3 proteins, and based on conservation of synteny between zebrafish *wnt3* and known *Wnt3* genes in mouse, human, and fugu, we propose that we have identified the zebrafish *wnt3* orthologue. Surprisingly, zebrafish *wnt3* does not appear to be expressed prior to the beginning of somitogenesis, making it highly unlikely that Wnt3 participates in specification of mesoderm, as has been shown for mouse Wnt3 (Liu et al., 1999). This difference should permit the study of later roles for Wnt3 in embryological processes such as neural patterning, which have been refractory to study by targeted deletion in mouse.

Zebrafish *wnt3* is expressed in embryos beginning at the tailbud stage in the developing neural plate and the tailbud. *wnt3* expression in bilateral stripes near the finbud progenitors marked by *fgf24* during somitogenesis is intriguing. The expression appears to be medial to the developing finbud fields and is transient. Future studies should address whether Wnt3 helps in establishing the finbud field. We were not able to detect *wnt3* in the finbuds at later time points, making it unlikely that zebrafish Wnt3 regulates AER outgrowth as observed in mouse (Barrow et al., 2003). One possibility is that *wnt3l*, which is expressed later in the finbuds proper (Norton et al., 2005), substitutes for *wnt3* in zebrafish AER regulation.

Neural expression of *wnt3* closely parallels that seen in other vertebrates (Roelink et al., 1990; Roelink and Nusse, 1991; Salinas and Nusse, 1992; Bulfone et al., 1993; Parr et al., 1993; Braun et al., 2003; Robertson et al., 2004), with notable expression in the diencephalon, dorsal mesencephalon, MHB, and dorsal hindbrain and spinal cord. In other vertebrates, Wnt3 is thought to be responsible for posteriorizing the forebrain, opposing the actions of transcription factors such as Six3 and Wnt antagonists such as Dkk1, and playing a role in formation of the ZLI (Braun et al., 2003; Wilson and Houart, 2004; Lewis et al., 2008). Wnt3 has also been suggested to be responsible for helping instruct the topography of retinal axon wiring to the optic tectum (Schmitt et al., 2006). The pattern of zebrafish *wnt3* expression makes it likely that these functions are conserved in fish. Knockdown of function using antisense morpholino oligonucleotides should help to elucidate how Wnt3 functions in

neural patterning across phyla. Interestingly, zebrafish *wnt3* alone among the known putatively canonical zebrafish Wnt genes is expressed in the basal plate. This expression may be indicative of some yet unknown role for Wnt signaling in patterning the tegmentum and/or ventral diencephalon.

EXPERIMENTAL PROCEDURES

Fish maintenance and microinjection

Zebrafish stocks (AB*, WIK, and *Tg*(*TOP:GFP*)$^{w25/+}$) were cared for and embryos were obtained through natural spawning and staged according to established procedures (Westerfield, 2000). 1-cell stage embryos were microinjected with the indicated masses of RNA in a volume of 1 nl (calibrated by injection of test drops into halocarbon oil series 27 (Sigma Aldrich, St. Louis, MO) on a stage micrometer), using a femtojet microinjector (Eppendorf, Westbury, NY).

RNA isolation, cDNA preparation, and RT-PCR

RNA was isolated from groups of 20 whole embryos at stages noted in the text using TRIzol (Invitrogen, Philadelphia, PA) according to the manufacturer's instructions. cDNA was generated from total RNA using the Superscript III RTPCR Kit (Invitrogen). PCR analysis of stage-specific cDNA was performed with Taq polymerase (Invitrogen) using 35-cycles. PCR products were analyzed on an agarose gel and were digitally recorded using a UVP BioDoc-It gel visualization system (UVP, Upland, CA). RT-PCR primers were previously described for *ef-1α* (Chen and Kimelman, 2000). New primers for *wnt3* RT-PCR were zWnt3-RT-F (5'-TACGCCTTCTTCAAGCATCC-3') and zWnt3-RT-R (5'-CTCTTTGCGCTTTTCTGTCC-3').

Constructs and mRNA synthesis

A putative *wnt3* homologue was identified as described in the text and amplified using 40 cycles of PCR with Taq (Invitrogen) from 24 hpf AB* embryo cDNA with the primers 5'Wnt3-UTR-F (5'-CCCCTCTCTAGGAATCCTATG-3') and zWnt3-R (5'-tctagaTTATTTACATGTATGTACGTCGTAGACC-3'), which introduces a 3' Xba1 site to facilitate subcloning. The 1137bp product was cloned to the pCRII vector (Invitrogen) using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Constructs were sequenced and compared to predicted sequence using the Sequencher software (GeneCodes Corp., Ann Arbor, MI). An amplified clone containing no non-silent polymorphisms compared to the Ensembl-predicted sequence was subcloned to pCS2+ (Turner and Weintraub, 1994) via EcoR1 and Xba1 (New England Biolabs (NEB), Ipswich, MA), to facilitate mRNA synthesis. The entire ORF and three 5' nucleotides of *shha* were amplified from 16-s. stage AB* cDNA using the forward primer 5'-ggatccAAAATGCGGCTTTTGAC-3' and reverse primer 5'-gaattcTCAGCTTGAGTTTACTGACATCC-3', which introduce 5' BamH1 and 3' EcoR1 sites to facilitate subcloning. The entire ORF and three 5' nucleotides of *fgf24* were amplified from 24 hpf AB* cDNA using the forward primer 5'-AAGATGTCTGTTCTGCCGTCAAG-3' and reverse primer 5'-tctagaCTCAGTTTGTATTGGGGTTGGG-3', which introduces a 3' Xba1 site. Wild-type clones were confirmed by sequencing. Amplicons were TOPO-TA cloned yielding pCRII +*shha* and pCRII+*fgf24*. pBS SK *chordin* was the generous gift of D. Kimelman. pGEM-T-Easy+*wnt3l* was the generous gift of R. Dorsky. pCS2+*dnFgfr1-gfp* was the generous gift of K. Poss. mRNA was synthesized from pCS2+*wnt3* following linearization with Not1 (NEB) using the mMessage mMachine Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Linearized template was degraded, mRNA was phenol extracted, then washed and concentrated in a Microcon YM-100 centrifugal filter (Millipore, Billerica, MA).

Genomic and phylogenetic analysis

Identification of a putative *wnt3* homologue was as described in the text. Deduced amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994). Aligned sequences were prepared for publication using the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic comparisons were performed using the PHYLIP software (http://evolution.genetics.washington.edu/phylip.html) by the neighbor-joining distance method with bootstrap analysis (100 replicates). Sequences used for comparison were mWnt3 (NP_033547), hWnt3 (NP_110380), zWnt3l (AAT38336), mWnt3a (NP_033548), and mWnt8a (NP_033316). Conservation of synteny was established by examination of neighboring genes using the Ensembl genome browser (http://www.ensembl.org).

In situ hybridization

Digoxigenin-labeled probe synthesis was as previously described (Clements and Kimelman, 2005). pCRII *wnt3* was linearized with Spe1 (NEB) and transcribed with T7 polymerase (NEB). Digoxigenin *gfp* probe was produced from pCS2+*dnFgfr1-gfp* linearized with BamH1 (NEB), which cuts immediately 5' to the *gfp* ORF, and transcribed with T7. For digoxigenin *fgf24*, pCRII+*fgf24* was linearized with Not1 (NEB) and transcribed with SP6 (NEB) For fluorescein- and digoxginenin-labeled *wnt3l* probe, pGEM-T-Easy+*wnt3l* was linearized with Sma1 (NEB) and transcribed with T7. Fluorescein-labeled *shha* probe was made from pCRII+*shha* linearized with Not1 and transcribed with SP6. Embryos older than 24 hpf were raised in 1-Phenyl-2-thiourea (PTU; Sigma Aldrich) to prevent pigmentation. Single and two-color WISH was performed according to established protocols, essentially as described (Oxtoby and Jowett, 1993; Clements and Kimelman, 2005). Embryos were mounted in 3% methyl cellulose (Sigma Aldrich) for photography.

Acknowledgments

We thank Dr. David Kimelman and Dr. Kenneth Poss for probe constructs. We thank Dr. Richard Dorsky for probe constructs and transgenic animals. We thank Dr. Chris Thorpe for valuable discussion of Wnt3. We thank Dr. Joshua Waxman for advice on two-color WISH. We thank Dr. Carole Weaver for critical evaluation of the manuscript and moral support. This work was supported by an American Heart Association postdoctoral fellowship to W.K.C. (0725086Y) and NIH R01-DK074482 to D.T.

Grant Sponsor: NIH R01-DK074482 to D.T. and American Heart Association award 0725086Y to W.K.C.

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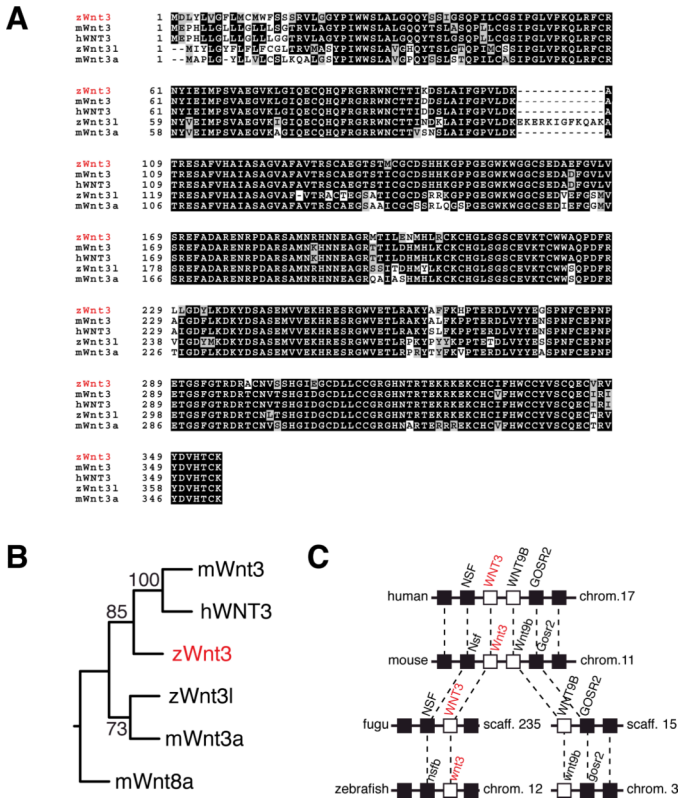

Fig. 1.

Zebrafish *wnt3* is the orthologue of other vertebrate *Wnt3* genes. **A:** Sequence conservation of the deduced amino acid sequence for zebrafish Wnt3, comparing mouse Wnt3 (mWnt3, top), human WNT3 (hWNT3, second from top), zebrafish Wnt3l (zWnt3l, second from bottom), and mouse Wnt3a (mWnt3a) bottom. **B:** Phylogenetic analysis of zWnt3 compared to similar genes in zebrafish mouse and human. zWnt3 clusters with known human and mouse Wnt3 genes, whereas *zwnt3l* clusters with mWnt3a. mWnt8a was used as the outgroup. Numbers indicate bootstrap confidences at branch points. **C:** Conservation of synteny between *Wnt3* genes in human, mouse, fugu, and zebrafish. The *Nsf* gene is the nearest 3'-neighbor in all four species.

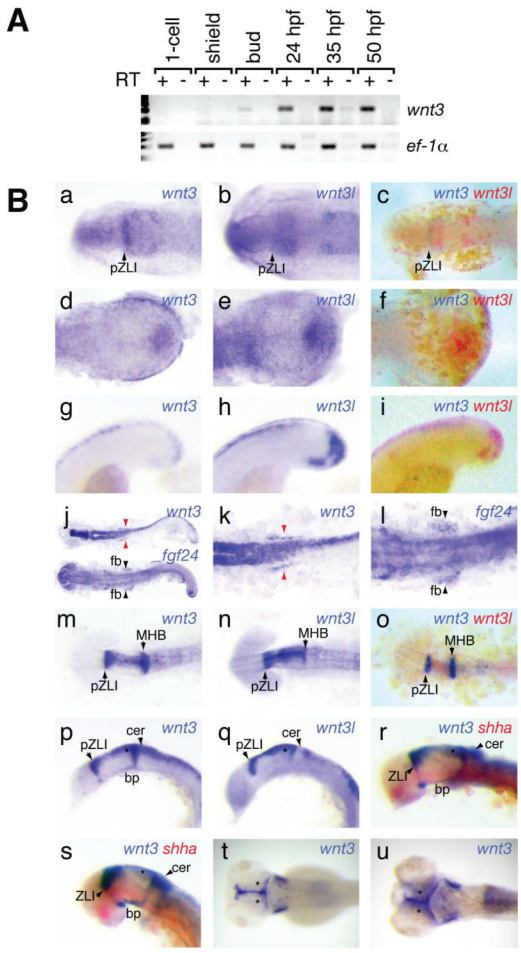

Fig. 2.

Temporal and spatial expression of *wnt3* during zebrafish embryonic development. **A:** RT-PCR analysis of *wnt3* (top row) in total cDNA from whole embryos at the indicated stages of development. *ef-1α* was used as a positive control. **B:** WISH analysis of *wnt3* expression. Embryos at various ages processed for expression of the mRNAs indicated. In all cases anterior is to the left. Flatmounted head views at 3-s. (a–c) show that *wnt3* displays stronger expression in the presumptive ZLI (black arrows, pZLI), whereas *wnt3l* displays stronger expression in the prospective midbrain just posterior. Flatmounted tailbud views at 3-s. (d–f). Note *wnt3* shows stronger expression in the lateral and posterior border of the tailbud. Lateral views of the tailbud at 18-s. (g–i). *wnt3l* shows stronger and deeper expression in the tailbud. Comparative flatmount views of *wnt3* and *fgf24* at 22-s. (j) and magnified images at the level of the finbud progenitors (k, l). *wnt3* is expressed bilaterally (red arrowheads) in tissue just medial to the finbud progenitor fields marked by *fgf24* (black arrowheads, fb). Dorsal flatmount views at 18-s. (m–o). Both *wnt3* and *wnt3l* are expressed in the developing diencephalon and mesencephalon, but *wnt3* is expressed farther posterior into the presumptive cerebellum. Lateral views of the brainstem with eyes removed at 18-s. (p–r) confirm *wnt3* expression into the presumptive cerebellum and demonstrate that *wnt3* extends much farther ventrally at both the presumptive ZLI and the midbrain hindbrain boundary, reaching into the presumptive basal plate. At 24 and 30 hpf (r, s; lateral brain stem views, eyes removed), this trend continues. Double WISH with *shha* confirms the basal plate expression. At 4 dpf (t) and 6 dpf (u) *wnt3* continues to be expressed in the optic tectum, and is visible in the otic vesicles (dorsal head views). Anatomical landmarks: bp, basal plate; cer,

cerebellum; fb, finbud progenitors; MHB, midbrain-hindbrain boundary; pZLI, presumptive *zona limitans intrathalamica*; ZLI, *zona limitans intrathalamica*; * optic tectum.

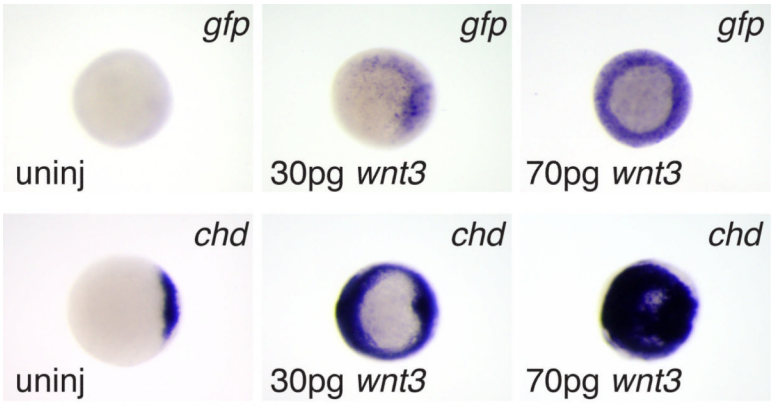

Fig. 3.

Zebrafish Wnt3 can activate the canonical Wnt pathway. Zebrafish heterozygous for a *gfp* transgene under the control of a Wnt/β-catenin-dependent promoter (*Tg*(*TOP:GFP*)$^{w25/+}$) were outcrossed, and the resulting embryos were allowed to develop uninjected (left column), or injected with either 30 pg (middle column) or 70pg (right column) of *wnt3* mRNA. At shield stage, embryos were fixed and processed by WISH for expression of either *gfp* (top row) or *chd* (bottom row) as indicated. Injected embryos showed a dose-dependent increase in canonical Wnt signaling-driven transcript abundance. 100% of embryos displayed the phenotypes depicted for each condition. Non-transgenic siblings provided an internal control for *gfp* in situs (not pictured).