Receptor Tyrosine Kinases ror1/2 and ryk Are Co-expressed with Multiple Wnt Signaling Components During Early Development of Sea Urchin Embryos

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Abstract. A combination of receptors, co-receptors, and secreted Wnt modulators form protein complexes at the cell surface that activate one or more of the three different Wnt signaling pathways (Wnt/β-catenin, Wnt/INK, and Wnt/Ca2+). Two or more of these pathways are often active in the same cellular territories, forming Wnt signaling networks; however, the molecular mechanisms necessary to integrate information from these pathways in these situations are unclear in any in vivo model system. Recent studies have implicated two Wnt binding receptor tyrosine kinases, receptor tyrosine kinase-like orphan receptor (Ror) and related-to-receptor tyrosine kinase (Ryk), in the regulation of canonical and non-canonical Wnt signaling pathways, depending on the context; however, the spatiotemporal expression of these genes in relation to Wnt signaling components has not been well characterized in most deuterostome model systems. Here we use a combination of phylogenetic and spatiotemporal gene expression analyses to characterize Ror and Ryk orthologs in sea urchin embryos. Our phylogenetic analysis indicates that both ror1/2 and ryk originated as single genes from the metazoan ancestor. Expression analyses indicate that ror1/2 and ryk are expressed in the same domains of many Wnt ligands and Frizzled receptors essential for the specification and patterning of germ layers along the early anterior-posterior axis. In addition, both genes are co-expressed with Wnt signaling components in the gut, ventral ectoderm, and anterior neuroectoderm territories later in development. Together, our results indicate that Ror and Ryk have a complex evolutionary history and that their spatiotemporal expression suggests that they could contribute to the complexity of Wnt signaling in early sea urchin embryogenesis.

Introduction

Wnt signaling is an important cell-to-cell communication mechanism used by animals to orchestrate many cellular events in the embryo and adult body. There are at least three Wnt signaling pathways recognized thus far, designated as the canonical Wnt/β-catenin as well as the non-canonical Wnt/JNK (or PCP) and Wnt/Ca2+ pathways. The latter two pathways are termed the non-canonical pathways because they were identified after the Wnt/β-catenin pathway and are less characterized at the molecular level compared to the Wnt/β-catenin pathway (for review, see Amerongen and Nusse, 2009). Each of these pathways has been shown to be critical for the large array of developmental mechanisms involved in cell fate determination, morphogenesis, and cell polarity (Kühl et al., 2000; Yamanaka et al., 2002; Clark et al., 2012; Steinhart and Angers, 2018). Importantly, some recent studies indicate that
et al. promiscuous, with either several Wnts capable to bind a single Fzd receptor or, conversely, one Wnt interacting with multiple Fxds (Hsieh et al., 1999b; Carmon and Loose, 2010). Adding to the complexity of Wnt signaling, several recent studies indicate that two or more Wnt pathways are active in the same cell and/or territory, depending on the context, forming Wnt signaling networks (Hsieh et al., 1999b; Kestler and Kühl, 2008; Carmon and Loose, 2010; Range et al., 2013; Steinhart and Angers, 2018). However, the exact molecular mechanisms that integrate information from the different pathways are unclear. One important area of focus is that the Wnt components at the cell surface—a combination of secreted Wnt ligands, Fzd receptors and co-receptors (e.g., Lrp5/6) at the extracellular surface, and at the intracellular surface (e.g., Disheveled, Casein Kinase 1)—appear to form multimeric complexes that interact to activate a particular Wnt pathway (Hsieh et al., 1999b; Tamai et al., 2004; Carmon and Loose, 2010; Green et al., 2014; Steinhart and Angers, 2018). Yet it is still unclear how these membrane-localized complexes are able to activate the different Wnt intracellular cascades that influence transcriptional activity and/or cytoskeletal structures (Kestler and Kühl, 2008; Steinhart and Angers, 2018). Thus, it is essential that we have a more comprehensive view of the spatiotemporal expression of not only the Wnt ligands and Fzd receptors but also the co-receptors that could influence how each of these Wnt pathways is activated.

In addition to Fzd and the well-characterized co-receptor Lrp5/6, several other receptors and co-receptors are known to modulate canonical and non-canonical Wnt signaling in certain contexts (Billiard et al., 2005; Macheda et al., 2012; Joiner et al., 2013; Yaguchi et al., 2016; Roy et al., 2018). These include a subfamily of receptor tyrosine kinase (RTK) proteins, such as receptor tyrosine kinase-like orphan receptor (Ror) and related-to-receptor tyrosine kinase (Ryk) (Roy et al., 2018). Recent studies on these two Wnt binding receptors are widening the breadth of Wnt signaling research, and they present new opportunities for understanding the roles of Wnt signaling in embryonic development and disease (Amerongen et al., 2008; Green et al., 2014; Roy et al., 2018). Both of these receptors are able to bind Wnt ligands, but they do so through different extracellular Wnt binding domains. Ror proteins bind Wnts with a cysteine-rich domain (CRD) that bears close homology to the Wnt binding CRDs of Fzd receptors and secreted Frizzled-related proteins (sFRPs) (Rehn et al., 1998; Saldanha et al., 1998; Hikasa et al., 2002; Green et al., 2007). In contrast, several Ryk homologs have been shown to bind Wnts directly through a CRD that is more similar to the one contained in the secreted Wnt signaling antagonist Wnt inhibitory factor-1 (WIF-1) (Hsieh et al., 1999a; Yoshikawa et al., 2003; Blakely et al., 2013; Duan et al., 2017). As RTKs, a possible mechanism by which Ror and Ryk could propagate Wnt signals would be through phosphorylation of downstream targets, as seen in many other RTKs (Neben et al., 2019). However, it is unclear how these Wnt receptors and co-receptors transduce the intracellular signal, because the catalytic activity of Ror and Ryk appears to be compromised in some animals as a result of mutations at critical amino acid residues in their protein tyrosine kinase (PTK) domain (Katso et al., 1999; Mikels et al., 2009; Bainbridge et al., 2014). This has led some researchers to propose that these are pseudokinases (Mendrola et al., 2013). Importantly, the extent to which these mutations are conserved across metazoans has not been extensively investigated. Thus, a detailed comparison of Ror and Ryk PTK sequences among diverse metazoan species would inform our understanding of their origins and how they may have changed throughout evolution.

Studies in Drosophila, Caenorhabditis elegans, and vertebrates implicate Ror in various processes that mostly center around the non-canonical Wnt/JNK (or Wnt/PCP) signaling pathway (Green et al., 2014; Roy et al., 2018). In Xenopus, zebrafish, and mice, Ror2 mutants display characteristic Wnt/JNK phenotypes that include defects in cell movements, such as convergent extension, hair cell orientation, and directed cell migration (Hikasa et al., 2002; Nishita et al., 2006; Schambony and Wedlich, 2007; Bai et al., 2014). It has also been reported that Wnt5A and Ror2 work together in cell culture to antagonize Wnt/β-catenin signaling mediated by Wnt3A (Mikels and Nusse, 2006b). Interestingly, both ligands appear to use the same Fzd receptor (Schambony and Wedlich, 2007; Nishita et al., 2010), suggesting that Ror2 may influence the level of activation of each signaling pathway in these cells. Also, Ryk appears to predominantly modulate Wnt/β-catenin signaling pathways. For instance, it can interact with the core PCP signaling machinery (e.g., Vangl) (Andre et al., 2012) and is necessary for Wnt/JNK-dependent convergent extension movements during zebrafish and Xenopus development (Kim et al., 2008). In addition to Wnt/JNK signaling, Ryk has been shown to activate Wnt/Ca\(^{2+}\) signaling though interaction with Wnt ligands (Cheyette, 2004). As with Wnt/Ror signaling, there are also some reports that Ryk can modulate Wnt/β-catenin signaling in different cellular contexts (Mikels and Nusse, 2006a; Mikels et al., 2009; Witte et al., 2010; Berndt et al., 2011; Adamo et al., 2017); but the mechanism(s) is unclear. Finally, both Ror and Ryk can serve as bona fide Wnt receptors and directly bind Wnts to regulate both canonical and non-canonical Wnt signaling (Billiard et al., 2005; Yamamoto et al., 2008; Enomoto et al., 2009; Macheda et al., 2012; Bai et al., 2014; Roy et al., 2018). Together, these studies illustrate that Ror and Ryk provide context at the membrane that can modulate the activity of all three Wnt signaling pathways, depending on the context.

Sea urchin embryos are an important developmental model system for Wnt signaling network studies. They are marine invertebrates that belong to the phylum Echinodermata and that, along with hemichordates (acorn worms), form sister phyla (Ambulacaria). Importantly, these phyla share a common ancestor with chordates in the deuterostome clade (echinoderms,
hemichordates, amphioxus, tunicates, and vertebrates) (Cameron et al., 2000; Swalla and Xavier-Neto, 2008; Cannon et al., 2014). Sea urchin genomes include a large set of the ancestral Wnt signaling system components, including 11–12 Wnt ligands and 4 Fzd receptors, as well as several co-receptors, intracellular and extracellular modulators, and transcriptional effectors (Cui et al., 2014; Robert et al., 2014, 2019). During early sea urchin embryogenesis, there are at least two Wnt signaling phases involving Wnt signaling: (1) the anterior-posterior (AP) specification and patterning of the early germ layers (endoderm, mesoderm, equatorial ectoderm, and anterior neuroectoderm [ANE]) during cleavage and blastula stages, and (2) the subsequent patterning of territories between these germ layers, as well as regulating cell movements, during gastrulation (Cui et al., 2014; Martínez-Bartolomé and Range, 2019).

During the first phase, a Wnt signaling network involving all three characterized Wnt signaling pathways is used to specify and position the early germ layer gene regulatory networks (GRNs) along the AP axis (Range et al., 2013; Range, 2014, 2018; Range and Wei, 2016; Khadka et al., 2018; Martínez-Bartolomé and Range, 2019). Initially, Wnt/β-catenin signaling specifies the endomesodermal territory and downregulates the ANE GRN within posterior blastomeres by the 32-cell stage (Wikramanayake et al., 1998; Logan et al., 1999; Range et al., 2013). Then, nuclear β-catenin (nβ-catenin) activates expression of two Wnt ligands (Wnt1 and Wnt8) that act as a relay mechanism to initiate Fzd5/8-JNK signaling in more anterior ectodermal cells. This Wnt/JNK signaling pathway downregulates the ANE GRN around the equator of the embryo (Range et al., 2013). Finally, another non-canonical Wnt pathway mediated by Fzd1/2/7-PKC (possibly a Wnt/Ca2+ pathway) attenuates the level of both Wnt/β-catenin and Wnt-Fzd5/8-JNK signaling during this process. Together, this balanced Wnt network precisely patterns the early AP axis, and the different pathways are often active in the same cellular territories (Range et al., 2013; Range, 2018). Importantly, this AP Wnt network is one of the few examples of the three Wnt signaling pathways working on the same developmental process, and aspects of this AP Wnt network appear to be conserved in many deuterostomes, including vertebrates (Range, 2014).

The second phase of Wnt signaling is observed in the embryo during late blastula and gastrula stages. Most wnt genes are expressed in patterns that overlap with one or more of the four Fzd receptors expressed during these stages (Cui et al., 2014; Robert et al., 2014; Range, 2018). These expression patterns create a remarkably complicated Wnt signaling landscape. Thus far, Wnt1, Wnt6, Wnt8, and Wnt16 and Fzd5/8 and Fzd1/2/7 receptors are the Wnt components whose function has been shown to be required for gastrulation (Wikramanayake et al., 2004; Croce et al., 2006, 2011; Lhomond et al., 2012; Sethi et al., 2012; Range et al., 2013; Cui et al., 2014; Martínez-Bartolomé and Range, 2019). However, it is unclear which Wnt ligands interact with a particular receptor and/or co-receptor during gastrulation. Thus, an analysis of the role and Ryk expression could also give us a better foundation for understanding how the various Wnt pathways are selectively activated during these latter stages of development.

In this study we provide a detailed spatiotemporal expression analysis of Ror and Ryk orthologs in invertebrate deuterostome embryos. The goal here is to gain a broader understanding of how these Wnt receptors and co-receptors may interact with other extracellular components of the Wnt signaling networks that govern early sea urchin embryogenesis. We also investigated the evolution of these proteins by comparing phylogenetic relationships, protein domain architecture, and PTK subdomain sequences from echinoderms and a diverse set of metazoan species. This study serves as a basic foundation for future studies on the functions of Ror and Ryk as possible Wnt receptors and co-receptors in sea urchin embryos, as well as how they contribute to the evolution in other deuterostome embryos.

Materials and Methods

Animals and embryo cultures

Adult *Strongylocentrotus purpuratus* (Stimpson, 1857) urchins were obtained from Monterey Abalone Company (Monterey, CA), Marinus Scientific (Longbeach, CA), and Point Loma Marine Invertebrate Lab (Lakeside, CA). *Lumbriculus variegatus* (Müller, 1774) adults were obtained from Duke Marine Lab (Beaufort, NC) and Pelagic (Sugarloaf Key, FL). To obtain gametes, urchins were induced to spawn by shaking or by injecting with 0.5 mol L⁻¹ KCl into the body cavity. Eggs were fertilized, and embryos were subsequently raised in artificial seawater at 15 °C for *S. purpuratus* and at 22 °C for *L. variegatus* until desired embryonic stages.

RNA extraction and cDNA synthesis

The RNA from embryos at different times of development was extracted and purified by using an RNeasy Plus mini kit (Qiagen, Hilden, Germany). Purified RNA was reverse transcribed to cDNA by using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and random primers for the reverse transcription polymerase chain reaction (RT-PCR) kit. Total RNA samples for cloning and quantitative polymerase chain reaction (qPCR) were treated with DNase I from a DNA-free kit (Invitrogen) to remove any possible residual genomic DNA. The cDNA from embryos at the stage of 0–24 hours after fertilization was used to obtain clones for *ror1/2* and *ryk*. The cDNA amplifications were done using the following primers, modified via addition of the Sp6 polymerase promotor sequence on the 5' end of reverse primers: *ror1/2* forward: 5'-GCATAAGCTGCAATGATAA-3', *ror1/2* reverse: 5'-TGATTTTCTGTTGTAGGGC-3', *ryk* forward: 5'-CGAGAGCTACAAATTAC-3', *ryk* reverse: 5'-CATC ATGGTCTCCACTTTC-3'.

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Quantitative polymerase chain reaction

Appropriately staged embryos were collected and frozen in pre-chilled −80 °C ethanol. Total RNA extraction was performed as described above, and cDNA synthesis was done using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA); qPCR was performed as described (Wei et al., 2009), using SYBR green reagent (Bio-Rad Laboratories, Hercules, CA) and an ABI 7500 RT-PCR system (Applied Biosystems), with two batches and triplicate reactions for all embryonic stages analyzed. Primers used were as follows: ror1/2 forward: 5'-GTATAAGAAACGACGCCCTAC-3', ror1/2 reverse: 5'-TAGTACCTGTATCGGTGG-3', ryk forward: 5'-GGTGAAGAGATGTGCTTCCTCC-3', ryk reverse: 5'-TGGACATAGATCAGAATGTTGA-3'.

Colorimetric whole-mount in situ hybridization

Antisense RNA probes were synthesized with Sp6 polymerase enzyme (Roche, Basel, Switzerland), precipitated, and purified with an Illustra RNA purification kit (GE Healthcare, Chicago, IL). Alkaline phosphatase reporter whole-mount in situ hybridization (WMISH) was carried out as previously described (Wei et al., 2009; Sethi et al., 2014; Erkenbrack et al., 2019).

In situ hybridization chain reaction (HCR) buffers, amplifiers, and DNA probes for fzd1/2/7, fzd5/8, ror1/2, and ryk were designed by and purchased from Molecular Instruments (Los Angeles, CA). In situ HCR was conducted following a modified protocol from Choi et al. (2016). Lytechinus variegatus embryos were fixed in 4% paraformaldehyde in artificial seawater with 0.1% Tween for 1 hour at room temperature. Embryos were post-fixed in 100% methanol for 1 hour. Each probe was added to hybridization buffer at a concentration of 0.4 pmol and incubated for 18 hours at 37 °C. Amplifier hairpins that correspond to each probe were added at a concentration of 6 pmol per amplifier hairpin and incubated at room temperature in the dark for 18 hours. Wash steps were conducted using buffers from Molecular Instruments and 5 x saline-sodium citrate Tween (SSCT) buffer. Embryos were imaged immediately upon protocol completion to ensure that no degradation of the fluorescent signal had occurred.

Phylogenetic trees and protein sequence analysis

National Center for Biotechnology Information (NCBI) databases, using BLAST (Altschul et al., 1990) with human ROR1 and RYK amino acid sequences as queries, were searched to obtain Ror and Ryk amino acid sequences. To avoid incorrectly annotated sequences in the database, the identity of the retrieved sequence (selected initially based on the lowest e-value) was confirmed using the NCBI Conserved Domains tool (Savant-Bhonsale et al., 1999). Only sequences that contained both conserved intracellular and extracellular domains were used for further analysis. Sequence ID numbers of all analyzed sequences are available in Table A1.

For phylogenetic analyses, multiple sequence alignments were performed using MAFFT version 7 (Katoh et al., 2019) online service, using iterative refinement strategy E-INS-I. Poorly aligned regions were trimmed using MEGA (Stecher et al., 2020) for improved analysis. To calculate the phylogenetic trees, maximum likelihood phylogenies were generated using IQ-Tree version 1.6.12 as implemented in the IQ-Tree Web Server (Trifinopoulos et al., 2016), and branch support of generated trees was calculated using the ultra-fast bootstrap method (Minh et al., 2013). For easier readability, resulting trees were manipulated using FigTree version 1.4.2 (Rambaut, 2007) and colored using Adobe Photoshop 2020.

Results

Phylogenetic analysis of Ror and Ryk proteins in metazoans

To determine the phylogenetic distribution of Ror and Ryk homologs across animal taxa, we performed careful in silico searches of Ror and Ryk homologs from cnidarians to vertebrates. We identified Ror and Ryk protein sequences from 25 and 23 species, respectively, selected across diverse taxa; then the orthology of these sequences was explored with phylogenetic analysis, using maximum likelihood. We found one Ror homolog in the majority of the species surveyed, including most protostomes and invertebrate deuterostomes. In addition to the one Ror protein in insects, we identified a sequence in this group called Ror2, or sometimes termed “neurospecific receptor tyrosine kinase” (Nrk). However, as previously recognized, this protein is more closely related to another RTK, muscle-skeletal receptor protein tyrosine kinase (MusK), than to true Ror proteins (Sossin, 2006; Nye et al., 2020). Our phylogenetic assessment of Ror-related proteins in insects with Ror and MusK sequences across metazoans supports this conclusion (Fig. A1A). In addition, our analysis of Ror sequences confirmed the existence of two Ror paralogs in vertebrates, Ror1 and Ror2 (Fig. 1A). Outside of this group, we found two distinct Ror sequences in the tunicates Ciona intestinalis and Phallusia mammillata. Although these sequences are annotated as Ror1 and Ror2 in NCBI databases, we found that they do not cluster with the vertebrate Ror1 and Ror2 sequences. Instead, tunicate Ror1 and Ror2 form their own subgroups, with Ror1 and Ror2 of C. intestinalis grouping with Ror1 and Ror2 of P. mammillata, respectively (Fig. 1A). The Ror proteins found in other deuterostomes, including the cephalochordates Branchiostoma belcheri and Asymmetricus lucayanum, branch at the base of the two vertebrate Ror1 and Ror2 sequences, suggesting that these Ror paralogs are specific to vertebrates. Collectively, these results indicate that the metazoan common ancestor likely had one ror ortholog that was duplicated in tunicates and then separately in vertebrates.
Next, we examined the phylogenetic relationships of Ryk homologs across metazoan species. Our analysis revealed that one ryk gene is present in most species, including cnidarians, non-arthropod protostomes, and deuterostomes. Interestingly, we found a non-transcribed ryk pseudogene in addition to the one functionally characterized ryk gene in vertebrates (Fig. 1B; Table A1). In a notable exception from the other organisms, we found 2–3 Ryk homologs in some arthropods. For example, a single Ryk protein was found in many non-insect arthropods, including the horseshoe crab, spiders, and Daphnia. In contrast, our analysis showed that two ryk genes are present in many insects and that they form two distinct clusters, a Derailed (Drl)/Doughnut (Dnt) and a Drl-2 cluster (Fig. 1B). It is important to note that all three Ryk homologs (Drl, Drl-2, and Dnt) are present only in flies. Together, these data suggest that, like ror, there was likely one ryk gene present in the metazoan common ancestor and that the appearance of the additional ryk genes in insects resulted from gene duplications after their split from other arthropod groups.

Comparisons of Ror and Ryk protein domain architecture

Most Ror proteins have an N-terminal immunoglobulin (Ig)-like domain, Wnt-binding CRD, Kringle (KR) domain, transmembrane (TM) segment, and a cytoplasmic PTK domain (Fig. 2A). While the sea urchin Ror1/2 protein has only one KR domain, it contains two tandem CRD and Ig-like domains as opposed to the single pair seen in vertebrates and many invertebrates (Fig. 2A). To investigate the potential origin of this feature in the sea urchin Ror homolog, Ror1/2, we compared Ror protein domains among protostomes and deuterostomes (Fig. 2A). Assessment of deuterostome Ror orthologs revealed that the incongruity in the sea urchin Ror1/2 extracellular domain is shared among other echinoderms as well as hemichordates, but not chordates or protostomes. Interestingly, the hemichordate Ror protein contains a tandem repeat of Ig-like, CRD, KR, Ig-like, CRD, and KR domains in its extracellular segment (Fig. 2A). These observations suggest that the entire ectodomain of the protein may have been duplicated as a result of a partial ror gene duplication event, possibly at the base of Ambulacraria. Additionally, after the split of the echinoderm and hemichordate lineages, the KR domains may have been lost in an early echinoderm ancestor. This is evidenced by the lack of a duplicate KR domain not only in sea urchins and sea stars but also in the feather star Ocyxcomanus japonicus, which belongs to the basal echinoderm class Crinoidea (Cannon et al., 2014).

Protein kinases share a core catalytic domain that is subdivided into 12 subdomains (numbered I–XII). Each of these subdomains contains conserved residues that are critical for catalytic activity (Carrera et al., 1993; Taylor et al., 1995; Hubbard and Till, 2000). Subdomains I, II, VIb, and VII are particularly important for binding adenosine triphosphate (ATP) and cation co-factors, such as Mg^{2+} (Hanks et al., 1988; Hubbard...
Subdomains I and II are critical for binding ATP, while subdomains VIb and VII make up the kinase active site and the magnesium-binding apparatus, respectively (Carrera et al., 1993; McClendon et al., 2014). Subdomains VIa–XI are important for coordinating the spatial interactions necessary to facilitate peptide-substrate interaction and for successful phospho-transfer (Hubbard and Till, 2000; McClendon et al., 2014). In vertebrates, Ror and Ryk have been proposed to be weak or inactive kinases as a result of an inability to bind ATP due to mutations in certain subdomains (Trivier and Ganesan, 2002; Nishita et al., 2006; Mendrola et al., 2013; Murphy et al., 2014).

To investigate the potential evolutionary origin of these mutations and their occurrence, or lack thereof, in sea urchins and other metazoans, we compared Ror and Ryk subdomains I, II, VIb, and VII in the sea urchins *Strongylocentrotus purpuratus* and *Lumbriculus variegatus* to those in a select number of deuterostomes and protostomes. The Ror PTK subdomains showed that vertebrate Ror1 and Ror2 proteins display mutations in subdomain I, but not in subdomains II, VIb, and VII (Fig. 2B), consistent with previous reports. In subdomain I, we observed a deviation in the glycine (G)-rich GxGxxG consensus motif, where the second glycine is substituted for cysteine (C), aspartate (D), or serine (S), depending on the paralog and species (Fig. 2B). Outside vertebrates, we observed deviations in tunicate as well as in cnidarian Ror proteins. In tunicates, Ror1 and Ror2 of *C. intestinalis* and Ror1 of *P. mammillata* share a common mutation that substitutes the original negatively charged aspartate (D) for an uncharged asparagine (N). In Ror2 of *P. mammillata*, we observed mutations in subdomains I and VII, where a serine (S) is observed in the place of the second G in subdomain I and a leucine (L) in the place of an aspartate (D) in subdomain VII. In *Nematostella vectensis* and *Exaiptasia diaphana*, substitutions are observed in subdomains II and VII. In subdomain II, the positively charged lysine (K) residue in the VAIK consensus motif is substituted for a valine (V) in *N. vectensis* and a serine (S) in *E. diaphana*. In subdomain VII, an alanine (A) is observed in the place of a glycine (G) in the DFG consensus motif in both cnidarian species (Fig. 2B). In contrast, we did not observe mutations in any of the analyzed subdomains in sea urchins or in the Ror proteins of other invertebrate deuterostomes and protostomes (Fig. 2B). These results indicate that Ror in vertebrates, tunicates, and cnidarians exhibits mutations that may render them catalytically inactive, while Ror in sea urchins and other metazoan species lacks these same mutations. Interestingly, the mutations observed in Ror proteins are highly lineage specific and occur in distinct positions in different lineages, indicating independent origins.

The Ror proteins consist of an extracellular Wnt inhibitory factor (WIF) domain, a transmembrane domain, and a cytoplasmic PTK domain (Fig. 2C). The sea urchin Ryk is highly conserved with other Ryk proteins, consisting of the same major domains (Fig. 2C). The Ryk protein has been previously...
suggested to lack intrinsic kinase function in vertebrates and in *Drosophila melanogaster* due to mutations in its kinase subdomains (Halford and Stacker, 2001;  Hing et al., 2020). To explore the potential kinase function of Ryk in sea urchins, we analyzed these subdomains in *S. purpuratus* and *L. variegatus*, as well as several other species, from cnidarians to vertebrates. We observed very similar mutations in subdomains I and VII in all the species analyzed. The subdomain I sequence of Ryk is altered such that the first glycine (G) in the GxGxxG consensus motif is substituted for a glutamine (Q) in *L. variegatus*, *Homo sapiens*, and *Mus musculus* and in Drl and Dnt of *D. melanogaster* (Fig. 2D). A leucine (L) is observed in this same position in *S. purpuratus* and a lysine (K) in Drl-2 of *D. melanogaster*. Two substitutions occurred in subdomain I of *C. intestinalis* and in the cnidarian *Pocillopora damicornis*. In *C. intestinalis*, we observed a leucine (L) and an alanine (A) in the place of the first and last glycine (G), respectively, while in *P. damicornis* a glutamate (E) and an alanine (A) are seen in the same respective positions (Fig. 2D). The mutations observed in subdomain VII of Ryk are incredibly conserved among deuterostome species. In this subdomain, the original DFG is replaced with DNA in all of the deuterostomes analyzed (Fig. 2D). In this same position, DFG is replaced with DNA in all of the deuterostomes anana-

**Spatiotemporal expression of ror1/2 and ryk during gastrulation**

As mentioned in the Introduction, information from at least three different Wnt signaling pathways is integrated to specify and pattern the early germ layer GRNs along the AP axis. To determine whether Ror and/or Ryk may be integrated into this critical developmental process, we analyzed their spatial expression. Using colorimetric WMISH, we showed that both transcripts were supplied maternally and uniformly in the egg (Fig. 3Ba, Ca; Fig. A2). *ryk* transcripts remained ubiquitously expressed during early cleavage stages while *ror1/2* was downregulated from the micromeres by the 60-cell stage (Fig. 3Bc, d, 3Cb–e). By the hatched blastula stage, *ror1/2* expression appeared ubiquitously expressed, with a slight upregulation on one side of the presumptive ectoderm, while *ryk* expression remained ubiquitously expressed (Fig. 3Bf, Cd–f). A few hours later, *ror1/2* was mostly downregulated within the ANE but remained expressed in the ventral (oral) equatorial ectodermal territory and in a ring of cells around the endoderm-endoderm boundary (Fig. 3Bg–j, inset). The gene *ryk* showed a similar expression pattern to that of *ror1/2* in the ventral ectoderm at the mesenchyme blastula stage (Fig. 3Ci–j, inset). In contrast to *ror1/2*, *ryk* was expressed throughout the endoderm, mesoderm, ventral ectoderm, and ANE at these stages, but downregulated from the dorsal ectoderm. Together, these data indicate that *ror1/2* and *ryk* are co-expressed in the same territories with multiple Wnt signaling network components that govern AP axis specification and patterning (summarized in Fig. 3A).
Figure 3. *ror1/2* and *ryk* mRNA is co-expressed with components of the anterior-posterior (AP) Wnt signaling network during cleavage and blastula stages. (A) Schematic diagram of *wnt* and *frizzled* (*fzd*) gene expression during early cleavage and blastula stages in sea urchins. (B, C) Spatiotemporal expression of *ror1/2* and *ryk* during cleavage, blastula, and very early gastrula stages in *Strongylocentrotus purpuratus*. (Ba–e) Ubiquitous expression of *ror1/2* mRNA from fertilization to early blastula stages. (Bf–j) Progressive restriction of *ror1/2* expression to the ventral ectoderm. (Ca–e) Ubiquitous expression of *ror1/2* mRNA from fertilization to early blastula stages. (Ci–j) Downregulation of *ryk* expression from the dorsal ectoderm in mesenchyme blastula and early gastrula staged embryos. ANE, anterior neuroectoderm; EB, early blastula; hpf, hours postfertilization; MB, mesenchyme blastula; PMC, primary mesenchyme cell; VV, vegetal view. Scale bars = 20 μm.
these stages. Collectively, these results present a detailed expression landscape of \textit{ror} and \textit{ryk} during early sea urchin embryogenesis, showing that these genes are co-expressed with a remarkable variety of Wnts and Fzds, depending on the embryonic stage.

\textbf{Figure 4.} \textit{ror1/2} and \textit{ryk} are expressed dynamically during gastrula and prism stages. (A) Diagram represents a summary of \textit{wnt} and \textit{frizzled} (\textit{fzd}) gene expression during gastrulation and later development in sea urchins. Data taken from Range et al. (2013), Cui et al. (2014), Robert et al. (2014, 2019), and Yaguchi et al. (2016). (B, C) Spatiotemporal expression of \textit{ror1/2} and \textit{ryk} in the gastrula and prism stage of \textit{Strongylocentrotus purpuratus}. (Ba–e) Restriction of \textit{ror1/2} expression to the ventral ectoderm, archenteron, and posterior lateral dorsal ectoderm. (Bf–j) Downregulation of \textit{ror1/2} in the foregut and midgut and upregulation in coelomic pouches. (Ca–e) Restriction of \textit{ryk} expression to the anterior neuroectoderm, ventral ectoderm, and archenteron. (Cf–j) Downregulation of \textit{ryk} in the midgut and upregulation in the coelomic pouches. ANE, anterior neuroectoderm; EG, early gastrula; hpf, hours postfertilization; LG, late gastrula; OV, oral view; PMC, primary mesenchyme cell; Pr, prism; SMC, secondary mesenchyme cell; VV, vegetal view. Scale bars = 20 \textmu m.

\textit{ror1/2} and \textit{ryk} are dynamically co-expressed with \textit{fzd1/2/7} and \textit{fzd5/8}

To date, the only functionally characterized Fzd receptors involved in early embryonic axis specification and patterning
in sea urchins are *fzd1/2/7* and *fzd5/8* (Croce et al., 2006; Range et al., 2013; Range, 2018). To explicitly examine the co-expression of *ror1/2* and *ryk* with these two *fzd* receptors, we conducted whole-mount *in situ* HCR (Fig. 5). At the mid-blastula stage, both *ror1/2* and *ryk* are ubiquitously expressed throughout the embryo, with *ryk* expression increased at the posterior (vegetal) side. At this stage *fzd1/2/7* and *fzd5/8* show incongruent expression in the ectodermal territory, with

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**Figure 5.** *ror1/2* and *ryk* are co-expressed dynamically with *fzd1/2/7* and *fzd5/8*. (A) *ror1/2* is co-expressed with both *frizzled* (*fzd*) receptors during the mid-blastula stage. *ror1/2* is downregulated in the ventral ectoderm during mesenchyme blastula and gastrula stages, where *fzd1/2/7* is upregulated and co-expressed with *fzd5/8* only in the mesoderm cells. (B) *ryk* is also co-expressed with both *fzd* receptors but shows increased expression in the posterior pole of the embryo during the mid-blastula stage. At the mesenchyme blastula stage, *ryk* maintains co-expression with both *fzd* receptors, but by the gastrula stage, it is excluded from embryonic territories overlapping with *fzd1/2/7* but maintains co-expression with *fzd5/8* in the anterior neuroectoderm. DAPI, 4′,6-diamidino-2-phenylindole.
fzd1/2/7 expressed in the equatorial ectoderm and fzd5/8 in the ANE, overlapping with both ror and ryk expression (Fig. 5A, B, top). By the mesenchyme blastula and gastrula stages, fzd1/2/7 has been shown to be upregulated in the ventral ectoderm, while fzd5/8 is maintained in the ANE and begins to be expressed in the mesoderm (Croce et al., 2006; Range et al., 2013; Robert et al., 2014; Range, 2018). At the mesenchyme blastula and gastrula stages, ror1/2 is downregulated in the equatorial ectoderm where fzd1/2/7 is expressed (Fig. 5A, middle), suggesting that fzd1/2/7 may be involved in the downregulation of ror1/2. Interestingly, at the mesenchyme blastula stages, ror1/2 is expressed in the same mesoderm cells as fzd5/8, indicating that ror1/2 may be involved in modulating the role of fzd5/8 in movements of gastrulation (Croce et al., 2006; Fig. 5A, middle). In contrast to ror1/2, ryk expression is maintained in the ANE as well as the ventral ectoderm, endoderm, and mesoderm territories. In addition, ryk appears to be completely downregulated in the dorsal ectoderm (Fig. 5B, middle). This expression pattern suggests that Ryk may play a role in the uncharacterized functions of both pathways at this stage. At the gastrula stage, ror1/2 was expressed only in the endoderm and mesoderm, while ryk appeared to be expressed in a territory abutting the blastopore and ectoderm-endoderm boundary, which overlaps with ror1/2 (Fig. 5A, B, third panels). Finally, ryk expression overlapped significantly with fzd5/8 at this stage (Fig. 5B, third panel). This sensitive HCR analysis clearly showed that both ror1/2 and ryk exhibit dynamic co-expression with these critical fzd receptors and that they suggest future avenues for functional studies.

Discussion

Recent studies in multiple animals indicate that the Ror and Ryk RTK receptors play important roles among the various Wnt signaling pathways, depending on the context. Here we present an analysis of the phylogenetic relationships, protein domains, and early spatiotemporal expression of these RTK receptors, with a focus on the sea urchin Strongylocentrotus purpuratus. The phylogenetic analysis showed that the ancestral origin of both Ror1/2 and Ryk in metazoans was a single gene. Interestingly, our analyses indicated that Ror1/2 duplicated independently in tunicates and vertebrates. In addition, we showed that insects had multiple Ryks but that most metazoans had only one copy. Our exploration of Ror1/2 and Ryk domains indicates that ror1/2 in ambulacrarians went through a partial gene duplication event that resulted in additional extracellular domains. Additionally, our analysis of Ror1/2 and Ryk PTK subdomains suggests that the catalytic activity of Ryk in most species, Ror1 and Ror2 in vertebrates, Ror1 and 2 in tunicates, and Ror1/2 in cnidarians might be compromised due to mutations in these critical subdomains. The mutations seen in Ror proteins occur at different positions and are not present in the majority of the species analyzed, including ambulacrarians, cephalochordates, and protostomes (Fig. A1B), suggesting independent origins. Finally, we showed that both ror1/2 and ryk homologs are expressed within territories that overlap with the early AP Wnt signaling network as well as within other developmental territories later in development where Wnt signaling has been shown to be critical for development.

Previous studies on Ror and Ryk have been performed in several well-established model organisms (Savant-Bhonsale et al., 1999; Matsuda et al., 2001; Inoue et al., 2004; Green et al., 2007; Duan et al., 2017; Nye et al., 2020). To our knowledge, a detailed phylogenetic analysis had not been performed among metazoans, and spatiotemporal developmental expression analyses of these two genes were lacking in invertebrate deuterostomes. Our data indicate that the metazoan common ancestor likely had a single copy of both ror and ryk genes. Interestingly, Ror showed duplications in the vertebrate and tunicate lineages that were of independent origins. In contrast, only a single Ryk homolog was present in most species, with the exception of several insects. Our results point to a duplication of the ancestral ryk gene in insects or an earlier arthropod ancestor to create the drl/dnt and drl-2 paralogs. Then the drl/dnt gene was again duplicated in flies, giving them three ryk paralogs. In vitro studies in mammals suggest that the Ror and Ryk PTK domain either has very weak or lacks kinase activity as a result of mutations in the kinase subdomain sequences of Ror and Ryk (Katso et al., 1999; Kamitori et al., 2005; Bainbridge et al., 2014). However, whether these mutations are widely converged among animals is unknown. Our study analyzes these subdomain sequences in vertebrates, but we expanded this knowledge to include five ambulacrarians, two tunicates, two cephalochordates, a variety of protostomes, including molluscs, and three cnidarians. We showed that Ror in most species lacks such inactivating mutations, except for tunicates and cnidarians. Notably, most of the mutations observed in these two groups occurred at different positions from vertebrates and from each other (Fig. 2B), suggesting that these mutations arose independently. In contrast, most of the mutations observed in Ryk occurred in the same positions in most animals (Fig. 2D), indicating that the ancestral Ryk protein likely also harbored these mutations.

Many studies indicate that both Ror and Ryk are involved in modulating the non-canonical Wnt signaling pathways (Harrison and Stacker, 2001; Minami et al., 2010; Green et al., 2014). As mentioned in the Introduction, early AP specification and patterning is governed by a Wnt signaling network that begins during cleavage and is completed by the beginning of gastrulation. Importantly, at least two non-canonical pathways are involved in this fundamental developmental process: the Wnt1/Wnt8-Fzd5/8 JNK and Wnt16-Fzd1/2/7-PKC signaling pathways (Range et al., 2013; Range, 2018; Martínez-Bartolomé and Range, 2019). Our whole-mount in situ HCR analyses indicate that both ror1/2 and ryk are initially broadly expressed throughout early cleavage and blastula stages.
overlapping with both \( fzd1/2/7 \) and \( fzd5/8 \) expression (Range et al., 2013; Robert et al., 2014). Our previous studies have shown that Fzd1/2/7 signaling is broadly active throughout the early embryo during these stages, where it antagonizes both the posteriorly localized Wnt/β-catenin pathway and the activity of Fzd5/8-JNK signaling in the anterior ectoderm (Range et al., 2013; Martínez-Bartolomé and Range, 2019). Thus, Fzd1/2/7 signaling activity overlaps with both of these different Wnt pathways in the same cells and/or territories. In addition, we found that the broadly expressed Wnt ligand, Wnt16, activates Fzd1/2/7 signaling, whereas the posteriorly localized Wnt1 ligand as well as Wnt8, which is initially expressed in the endomesoderm and then progressively moves into the anterior ectoderm, activate the broadly expressed Fzd5/8 receptor in anterior blastomeres (see Fig. 3A; Range et al., 2013; Martínez-Bartolomé and Range, 2019). Thus, during early AP specification and patterning, two different ligands specifically activate the Wnt/JNK pathway, whereas another activates a Wnt/PKC pathway within the same anterior ectodermal territories. It is possible that Wnt1 and Wnt8 specifically interact with the CRD of Fzd5/8 and that Wnt16 interacts with the Fzd1/2/7 CRD; however, it is tempting to speculate that additional interactions with either Ror1/2 and/or Ryk may provide the extracellular context for these ligands to activate either pathway within the same territories.

A different scenario occurs in posterior endoderm and mesoderm cells as well as the cells that form the boundary between the endoderm and the ectoderm during the cleavage and blastula stages: Wnt6 is necessary to maintain Wnt/β-catenin signaling in endodermal cells, and Wnt16 has been shown to antagonize Wnt/β-catenin signaling in these same cells (Croce et al., 2011; Martínez-Bartolomé and Range, 2019). Remarkably, both ligands appear to use the same Fzd1/2/7 receptor, indicating that intricate control at the extracellular and/or intracellular level is essential for modulating these two signaling pathways in endodermal cells. As with the previous scenario, both ror1/2 and ryk are expressed within the posterior endomesoderm during these early specification and patterning events, suggesting that they could play a role in modulating these apparent promiscuous Wnt signaling events. Finally, a Wnt ligand that is not directly involved in early Wnt network governing AP specification and patterning, Wnt5, begins expression in endodermal cells at the ectoderm-endoderm boundary during later blastula stages (McIntyre et al., 2013). Again, it is important to remember that during these stages only \( fzd1/2/7 \) and \( fzd5/8 \) are expressed, suggesting that Wnt5 must use the same receptors as Wnt1, Wnt8, and Wnt16 while activating different cellular mechanisms. Interestingly, our in situ HCR analysis suggests that ror1/2 is excluded from most of the endoderm while seeming to be upregulated in these boundary cells that respond to Wnt5 signaling. This expression pattern suggests that the Ror1/2 receptor may provide the context through which short-range Wnt5 is able to activate a specific set of genes at the ectoderm-endoderm boundary.

Together, these studies further strengthen the idea that extracellular co-receptors, such as Ror and Ryk, likely play critical roles in the multimeric extracellular protein complexes that activate specific Wnt signaling-dependent mechanisms, even within the same cells and territories.

The Wnt signaling landscape becomes more complex during the second phase of Wnt signaling that begins around the mesenchyme blastula stage in sea urchin embryos, especially within the posterior endoderm and mesoderm territories. As mentioned above, all four Fzd receptors and most Wnt ligands begin to be expressed in the endoderm and mesoderm territories during these stages (Range et al., 2013; Robert et al., 2014). To date, the specific Wnt/Fzd interactions that control the activation of particular GRNs and/or cytoskeletal rearrangements during these early gastrula stages are unknown. However, the readout for Wnt/β-catenin signaling, nβ-catenin, is observed in endomesoderm cells through the mid-blastula stages. Then it is progressively downregulated from the mesoderm as well as the most posterior ring of endoderm cells (Veg2) and stabilized within the most anterior ring of endodermal cells (Veg1) right before the invagination of the blastopore (Logan et al., 1999). The Wnt expression landscape (Fig. 3A) during these stages suggests that more than one Wnt/Fzd interaction could be involved in the progressive nβ-catenin shift toward the anterior and that this shift influences the activation of different endodermal and mesodermal GRNs. Consistent with this idea, different Wnt ligands have been shown to activate specific GRN networks within the endoderm territories around the beginning of gastrulation. For instance, Wnt1 and Wnt4 are co-expressed within the same vegetal cells in mesenchyme and early gastrula stage embryos (Robert et al., 2014) but activate different regulatory subcircuits (Sethi et al., 2012; Cui et al., 2014). The \( wnt1 \) gene is expressed earlier during endomesoderm specification and has been shown to be necessary for the expression of several regulatory factors in this territory (Sethi et al., 2012; Range et al., 2013; Cui et al., 2014). Interestingly, it is also necessary for a subset of regulatory factors (\( nkl, sp5, \) and \( unc4.1 ) \) in the most posterior ectodermal cells (Veg1 ectoderm) around the ectoderm-endoderm boundary. In contrast, Wnt4 is unnecessary for the expression of endodermal GRN components by mesenchyme blastula stages, and it activates a different set of transcription factors in the Veg1 ectoderm (\( unc4.1, hox7, \) and \( mxx ) \) (Cui et al., 2014). Another interesting example is Wnt16, whose broad activity is initially essential for Fzd1/2/7 signaling activity with the AP Wnt signaling network. Then it is subsequently essential for the expression of a very specific set of endodermal transcription factors, blimp1, eve, and hox11/13b, which are known to be necessary for gastrulation and morphogenetic movements of mesodermal cells (Arenas-Mena et al., 2006; Livi and Davidson, 2006; Cui et al., 2014; Martínez-Bartolomé and Range, 2019). Interestingly, Wnt16 expression progressively moves from posterior to anterior mesoderm and endodermal cells during late blastula
and early gastrula stages, overlapping with the expression of multiple Wnt ligands, including Wnt1 and Wnt4 (Martínez-Bartolomé and Range, 2019). To add to the complexity of Wnt signaling interactions within these posterior territories, the Fzd1/2/7 and Fzd5/8 receptors as well as Wnt1, Wnt8, Wnt6, and Wnt16 have been implicated in the morphogenetic movements of gastrulation (Wikramanayake et al., 2004; Croce et al., 2011; Wei et al., 2012; Martínez-Bartolomé and Range, 2019), which are generally thought to be controlled by the non-canonical Wnt pathways. Based on the overlapping expression of many Wnt ligands and Fzd receptors (Fig. 4A), it is likely that other co-receptors are necessary for modulating the Wnt signaling in these territories. Thus, the spatiotemporal expression of co-receptors provides critical clues to how this complex Wnt signaling environment might be regulated. It is interesting that ryk is expressed throughout the endodermal and mesodermal territories at the beginning of gastrulation, suggesting that it could be a member of one or more Wnt multimeric protein complexes necessary for pattern formation and/or morphogenetic processes in the central endoderm and mesodermal territories. In contrast, ror1/2 appears to be downregulated from the mesoderm and likely more posterior endodermal (Veg2) cells; however, ror1/2 appears to be expressed in an area corresponding to the more anterior endodermal cells (Veg1) and possible ectoderm at the ectoderm-endoderm boundary. Importantly, this is the same territory in which we see the restriction of Û–catenin to a ring of around two cells in the anterior endodermal cell (Veg1), suggesting a potential role for Ror1/2 in modulating canonical Wnt signaling there.

As gastrulation progresses, the spatiotemporal expression of the Wnt/Fzd landscape becomes more complex, as does ror1/2 and ryk expression. To date we have little functional understanding of how Wnt signaling might influence the later specification and patterning of the ANE territory. Thus, it is interesting that both ryk and fzd5/8 are expressed within the territories expressing the ANE GRN from cleavage to prism stages (Fig. 4A, C). Strengthening the idea that Wnt signaling may be involved in the ANE, expression studies also suggest that wnt1, wnt7, and wnt10 may also be expressed in this territory (Robert et al., 2014). Another interesting co-expression correlation is that ryk is expressed within the ventral equatorial ectodermal cells along with wnt8 and fzd1/2/7 during gastrula stages (Fig. 5). These are the only combination of extra-cellular Wnt signaling components that are co-expressed within these territories, and it is unclear how this combination of Wnt modulators may affect cell specification and patterning within either territory. Finally, both ror1/2 and ryk are expressed in the foregut and hindgut territories. This expression corresponds with a unique combination of fzd1/2/7, wnt4, and wnt9 in the foregut as well as fzd9/10 along with wnt1, wnt4, wnt7, wnt9, wnt10, and wnt16 in the hindgut (Fig. 4), suggesting another layer of possible Ror1/2, Ryk, and Wnt signaling interaction during early sea urchin development.

In conclusion, this study is an initial step toward understanding the role of Ror1/2 and Ryk in sea urchin embryos and, more broadly, metazoans as a whole. Few studies have focused on these important receptor tyrosine receptors in organisms outside of vertebrates, Drosophila, and Caenorhabditis elegans. Thus, this study will likely not only help us better understand the complex Wnt networks that govern early development of the sea urchin embryo but also provide a sound basis for future functional analyses on how Ror and Ryk may help us interpret how Wnt signaling networks are integrated at the extracellular surface in the future.

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Figure A1. Phylogenetic analysis of receptor tyrosine kinase-like orphan receptor (Ror) and muscle-skeletal receptor protein tyrosine kinase (Musk) protein sequences. (A) Maximum likelihood phylogeny of Ror and Musk sequences showing that insect Ror2 is likely not a true Ror protein, but more closely related to Musk proteins. (B) Alignment of the Ror kinase subdomains in ambulacrarians, cephalochordate, and diverse protostomes. ATP, adenosine triphosphate; PTK, protein tyrosine kinase.
Figure A2. Temporal expression of *ror1/2* and *ryk* in *Strongylocentrotus purpuratus* as measured by quantitative polymerase chain reaction.

| Species                        | Sequence ID       | Gene name (National Center for Biotechnology Information) |
|--------------------------------|-------------------|------------------------------------------------------------|
| Acropora digitifera            | XP_015759044.1    | ROR1-like                                                  |
| Actinia tenebrosa              | XP_031567701.1    | RYK                                                        |
| Actinia tenebrosa              | XP_031559843.1    | ROR1-like                                                  |
| Anoplophora glabripennis       | XP_018578936.1    | Derailed                                                   |
| Anoplophora glabripennis       | XP_018577842.1    | Doughnut-like                                              |
| Araneus ventricosus            | GBM06467.1        | RYK                                                        |
| Araneus ventricosus            | GBM09274.1        | ROR1                                                       |
| Asterias rubens                | XP_033638273.1    | RYK                                                        |
| Asterias rubens                | XP_033636995.1    | ROR1-like                                                  |
| Bicyclus anynana               | XP_023947589.1    | Doughnut-like                                              |
| Bicyclus anynana               | XP_023935339.1    | Ror                                                        |
| Bicyclus anynana               | XP_023948459.1    | Ror2                                                       |
| Branchiostoma belcheri         | XP_019640430.1    | ROR1-like                                                  |
| Branchiostoma floridiae        | XP_035692719.1    | RYK                                                        |
| Capitella teleta               | ELU14629.1        | CAPTEDRAFT_162027                                          |
| Ciona intestinalis             | XP_002130072.1    | ROR2                                                       |
| Ciona intestinalis             | XP_002130072.1    | ROR2                                                       |
| Danio rerio                    | XP_005165988.1    | RYK                                                        |
| Daphnia magna                  | EFX66645.1        | DAPPUDRAFT_307904                                          |
| Daphnia pulex                  | EFX68868.1        | DAPPUDRAFT_301267                                          |
| Dendronephthya gigantea        | XP_028401701.1    | RYK                                                        |
| Drosophila melanogaster        | NP_523705.2       | Otk                                                        |
| Drosophila melanogaster        | NP_612571.1       | Otk2                                                       |
| Drosophila melanogaster        | NP_001260567.1    | Doughnut                                                   |
| Drosophila melanogaster        | NP_001260566.1    | Derailed                                                   |
| Drosophila melanogaster        | NP_001286371.1    | Derailed-2                                                 |
| Drosophila melanogaster        | NP_476962.1       | Ror                                                        |
| Drosophila melanogaster        | NP_477255.1       | Nrk                                                        |
| Exaiptasia_diaphana            | XP_028514205.1    | ROR1                                                       |
| Gallus gallus                  | XP_015146426.1    | ROR1                                                       |
| Gallus gallus                  | XP_001074185.1    | ROR2                                                       |
| Homo sapiens                   | NC_000017.11      | RYK pseudogene 1                                           |
| Homo sapiens                   | NP_002949.2       | RYK                                                        |
| Homo sapiens                   | NP_690620.1       | PTK7                                                       |
| Homo sapiens                   | NP_005003.2       | ROR1                                                       |
| Species                        | Sequence ID       | Gene name (National Center for Biotechnology Information) |
|-------------------------------|-------------------|-----------------------------------------------------------|
| *Homo sapiens*                | BAD92391.1        | ROR2                                                      |
| *Homo sapiens*                | XP_005252051.1    | MUSK                                                      |
| *Limulus polyphemus*          | XP_013780639.2    | ROR1-like                                                 |
| *Mus musculus*                | EDL23512.1        | PTK7                                                     |
| *Mus musculus*                | NP_001036072.1    | RYK                                                       |
| *Mus musculus*                | NP_038873.2       | ROR1                                                      |
| *Mus musculus*                | NP_038874.3       | ROR2                                                      |
| *Musca domestica*             | XP_005181804.1    | Doughnut                                                  |
| *Musca domestica*             | XP_011295852.2    | Doughnut                                                  |
| *Musca domestica*             | XP_019895320.1    | Ror                                                       |
| *Nematostella vectensis*      | XP_032229628.1    | RYK-like                                                  |
| *Nematostella vectensis*      | XP_001633299.2    | PTK7                                                      |
| *Octopus bimaculoides*        | XP_014788082.1    | RYK-like                                                  |
| *Parasteatoda tepidariorum*   | XP_015930998.2    | RYK                                                       |
| *Phallusia mammillata*        | CAB3265672.1      | ROR1                                                      |
| *Phallusia mammillata*        | CAB3265673.1      | ROR2                                                      |
| *Platynereis dumerilli*       | ANS60436.1        | RYK                                                       |
| *Pocillopora damicornis*       | XP_027054963.1    | RYK                                                       |
| *Pocillopora damicornis*       | XP_027041304.1    | ROR1                                                      |
| *Pomacea canaliculata*        | XP_025080890.1    | ROR1-like                                                 |
| *Scaptodrosophila lebanonensis* | XP_030377378.1   | Doughnut                                                  |
| *Scaptodrosophila lebanonensis* | XP_030376744.1   | Derailed                                                  |
| *Scaptodrosophila lebanonensis* | XP_030377478.1   | Doughnut                                                  |
| *Stegodyphus mimosarum*       | KFM80785.1        | hypothetical protein                                      |
| *Strongylocentrotus purpuratus* | XP_030853345.1   | PTK7                                                      |
| *Strongylocentrotus purpuratus* | XP_030851448.1   | MUSK                                                      |
| *Strongylocentrotus purpuratus* | XP_030838359.1   | RYK                                                       |
| *Strongylocentrotus purpuratus* | XP_792459.2      | ROR1                                                      |
| *Stylophora pistillata*       | XP_027798224.1    | RYK                                                       |
| *Tribolium castaneum*          | XP_008194862.1    | Ror                                                       |
| *Tribolium castaneum*          | XP_008193390.1    | Ror                                                       |
| *Xenopus laevis*               | XP_018113729.1    | ROR1                                                      |
| *Xenopus laevis*               | XP_018099378.1    | ROR2-like                                                  |
| *Xenopus tropicalis*           | XP_012825929.1    | RYK                                                       |